Studies on the Regulatory Mechanisms of Fcγ Receptor Function

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DECLARATION

This thesis and the research described herein is solely my own work. All work presented in this thesis was, unless otherwise acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference. No part of this work has been, or is submitted for any other degree qualification.

Stylianos Bournazos

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11. Materials and Methods

11.1. Antibodies, Reagents and Culture Media

All chemical reagents were from Sigma Aldrich (Poole, Dorset, UK), unless otherwise stated. Human leukocyte elastase was from Elastin Products Company (Owensville, Missouri, USA) and dextran T-500 from Pharmacia / Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Percoll™ and OptiPrep™ density media were from GE Healthcare (Chalfont St. Giles, Bucks, Buckinghamshire, UK) and Axis Shield/Sigma Aldrich (Uxbridge, Middlesex, UK), respectively. Purified LPS (*E.coli* O111:B4) was from Sigma Aldrich and recombinant human TNF-α from R&D Systems (Abingdon, Oxfordshire, UK). Protease inhibitor cocktail reagents were from Roche Applied Science (Burgess Hill, West Sussex, UK) and Fluka / Sigma Aldrich (Buchs, Switzerland). Phosphatase inhibitors and the biotinylation reagent (EZ-Link™ Sulfo-NHS-LC-Biotin) were from Pierce / Thermo Fisher Scientific (Cramlington, Northumberland, UK). Protein G-Sepharose™ was from GE Healthcare. Ultrapure agarose was from Invitrogen (Renfrew, Paisley, Renfrewshire, UK) and low-melting point agarose was from Promega (Southampton, Hampshire, UK). Tris Borate EDTA (TBE) buffer was obtained from Invitrogen. FluorSave™ reagent was from Merck-Calbiochem (Beeston, Nottingham, UK). Fluorescein isothiocyanate- (FITC-), R-Phycoerythrin- (R-PE-) and allophycocyanin- (APC-) conjugated recombinant annexin V were obtained from Caltag, Invitrogen (Renfrew, Paisley, Renfrewshire, UK). TO-PRO®-3 nuclear stain, biotin-conjugated recombinant cholera toxin B subunit,

Alexa Fluor™ 647-conjugated recombinant streptavidin, dihydrorhodamine-123 (DHR123), 5-chloromethylfluorescein diacetate (CMFDA; CellTracker™ Green) and 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE)(CellTrace™ Far Red) were obtained from Molecular Probes, Invitrogen (Renfrew, Paisley, Renfrewshire, UK). Horseradish peroxidase (HRP)-conjugated recombinant streptavidin was from Dako Cytomation (Ely, Cambridgeshire, UK). Cholesterol and cholesteryl ester quantification kit was from Merck-Calbiochem. Cytometric bead array reagents (human inflammation kit) were from BD Biosciences (Oxford, Oxfordshire, UK).

Lipofectamine LTX™ and Lipofectamine PLUS™ transfection reagents were from Invitrogen and jetPEI™ reagent from PolyPlus™ Transfection (Autogen Bioclear, Calne, Wiltshire, UK). G418 (neomycin) was from Merck-Calbiochem or Invitrogen. Unless otherwise stated, all SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) reagents and buffers were from Invitrogen and Atto Corporation (Braintree, Essex, UK), with the exception of nitrocellulose protein transfer membrane, which was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Enhanced chemiluminescence (ECL™) and ECL Plus™ reagents were from Amersham/GE Healthcare (Chalfont St. Giles, Bucks, Buckinghamshire, UK). All PCR reagents were from Promega, with the exception of oligonucleotide primers, which were synthesised and purified by MWG Biotech – Eurofins (Ebersberg, Germany) and the high fidelity *Pfu* Polymerase, which was obtained from

Stratagene - Agilent Technologies UK (Stockport, Cheshire, UK). Reagents used for plasmid extraction (molecular biology- and cell transfection-grade), DNA extraction, nucleic acid purification, and qPCR were from Qiagen (Crawley, West Sussex, UK). Ligase and alkaline phosphatase enzymes were obtained from Roche Applied Science and restriction endonucleases were from Promega or Roche Applied Science.

The following mouse monoclonal anti-human CD32 antibodies were used in this study: IV.3 ($IgG2_b$, or $F(ab')_2$), AT-10 (IgG1, R-PE-conjugated, Abcam, Cambridge, Cambridgeshire, UK), or $F(ab')_2$), 7.3 ($F(ab')_2$ from IgG1, Ancell Corp. Bayport, Minnesota, USA), and FLI8.26 ($IgG2_b$, FITC-conjugated; BD Biosciences). Goat polyclonal anti-human CD32a (AF 1875) was obtained from R&D Systems. Corresponding isotype control antibodies were from Dako Cytomation, AbD Serotec (Kidlington, Oxfordshire, UK), or Sigma Aldrich and included: mouse monoclonal IgG1 isotype control (MOPC-21, Sigma Aldrich), mouse monoclonal $IgG2_a$ isotype control (UPC-10, Sigma Aldrich), mouse monoclonal $IgG2_b$ isotype control (MOPC-141, Sigma Aldrich), FITC- or R-PE-conjugated mouse IgG1 negative control (AbD Serotec). Mouse monoclonal anti-human caveolin-I (C060, IgM and 2297, IgG1), mouse monoclonal anti-human flotillin-1 (clone 18/flotillin-1, IgG1), mouse monoclonal anti-human transferrin receptor/CD71 (clone 2, IgG1), and biotin-conjugated mouse monoclonal anti-phosphotyrosine (PY-20, $IgG2_b$) were from BD Transduction Laboratories (BD Biosciences, Oxford, Oxfordshire, UK). FITC-conjugated mouse monoclonal anti-biotin (BN-34, IgG1), FITC-conjugated mouse

monoclonal anti-human CD16 (3G8, IgG1), FITC-conjugated mouse monoclonal anti-human CD62L (FMC46, $IgG2_b$), and agarose-conjugated goat polyclonal IgG anti-mouse immunoglobulin were from Sigma Aldrich. FITC-conjugated mouse monoclonal anti-human CD11b (ICRF44, IgG1), mouse monoclonal anti-human CD16 (3G8, IgG1), and mouse monoclonal anti-human CD64 (10.1, IgG1) were from AbD Serotec. FITC-conjugated mouse monoclonal anti-human CD24 (SN3, IgG1), and FITC-conjugated mouse monoclonal anti-human CD16 (3G8, IgG1) were from Caltag, Invitrogen and BD Pharmingen (Oxford, Oxfordshire, UK), respectively. R-PE-conjugated mouse monoclonal anti-human GPI-80 (3H9, IgG1) was obtained from MBL International (Woburn, Massachusetts, USA). CD16b allotype-specific mouse monoclonal antibodies were the following: for NA1: CLB-gran/11 ($IgG2_a$), and for NA2: GRM-1 ($IgG2_a$). HRP-conjugated goat polyclonal IgG anti-mouse immunoglobulin, HRP-conjugated rabbit polyclonal IgG anti-goat immunoglobulin, and FITC- or R-PE-conjugated goat polyclonal $F(ab')_2$ anti-mouse immunoglobulin were from Dako Cytomation. FITC- or Alexa Fluor™ 488-conjugated goat polyclonal $F(ab')_2$ anti-mouse immunoglobulin were from Invitrogen. Further details about all the antibodies used in this study are presented in *Table 11.1*.

Cell culture media, buffers and other supplements and reagents, including L-glutamine, penicillin/streptomycin and trypsin/EDTA solutions were obtained from PAA Laboratories (Pasching, Austria), unless otherwise stated. Iscove's Modified Dulbecco's Medium (IMDM) and RPMI 1640 media were from PAA

Laboratories or from Invitrogen. Dulbecco's Modified Eagle Medium (DMEM): Ham's F-12 (DMEM:F12) medium and Opti-MEM® I Reduced growth medium were obtained from Invitrogen. Foetal bovine serum (heat-inactivated or not) was purchased from Biowest (Ringmer, West Sussex, UK). Terrific Broth (TB), bacterial selection antibiotics and other reagents used for bacterial cell culture were from Sigma Aldrich. Lysogeny Broth (LB) media were prepared in-house with reagents purchased from Sigma Aldrich, BDH (VWR International, Lutterworth, Leicestershire, UK) and Oxoid (Basingstoke, Hampshire, UK).

Table 11.1: Monoclonal and Polyclonal Antibodies Used in this Study

Specificity	Reactivity	Host	Clonality	Isotype	Clone	Conjugation	Supplier
CD11b	Hu	Mo	Mono	IgG1	ICRF44	FITC (FL1)	AbD Serotec
CD16	Hu	Mo	Mono	IgG1	3G8	FITC (FL1)	BD Pharmingen
	Hu	Mo	Mono	IgG1	3G8	-	In house/Serotec
	Hu	Mo	Mono	IgG1	3G8	FITC	Sigma
CD16b NA1	Hu	Mo	Mono	IgG2a	CLB-gran/11	-	*1*
CD16b NA2	Hu	Mo	Mono	IgG2a	GRM-1	-	*1*
CD24	Hu	Mo	Mono	IgG1	SN3	FITC (FL1)	Invitrogen
CD32	Hu	Mo	Mono	IgG2b	FLI8.26	FITC (FL1)	BD Pharmingen
	Hu	Mo	Mono	IgG1	AT-10	R-PE (FL2)	Abcam
	Hu	Mo	Mono	$F(ab')_2$	AT-10	-	*2*
	Hu	Mo	Mono	$F(ab')_2$	7.3	-	Ancell
CD32a	Hu	Mo	Mono	IgG2b	IV.3	-	In house
	Hu	Mo	Mono	$F(ab')_2$	IV.3	-	In house
	Hu	Go	Mono	IgG	-	-	R&D Systems
CD62L	Hu	Mo	Mono	IgG2b	FMC46	FITC (FL1)	Sigma
CD64	Hu	Mo	Mono	IgG1	10.1	-	AbD Serotec
CD71	Hu	Mo	Mono	IgG1	2	-	BD Transduction
Caveolin-I	Hu, Ha	Mo	Mono	IgG1	2297	-	Laboratories
	Hu, Ha	Mo	Mono	IgM	C060	-	
Flotillin-1	Hu	Mo	Mono	IgG1	18	-	
GPI-80	Hu	Mo	Mono	IgG1	3H9	R-PE (FL2)	MBL Intl
Ig	Mo	Go	Poly	IgG	-	HRP	Dako Cytomation
	Go	Ra	Poly	IgG	-	HRP	Dako Cytomation
	Mo	Go	Poly	IgG	-	Agarose	Sigma
	Mo	Go	Poly	$F(ab')_2$	-	R-PE (FL2)	Dako Cytomation
	Mo	Go	Poly	$F(ab')_2$	-	FITC (FL1)	Dako Cytomation
	Mo	Go	Poly	$F(ab')_2$	-	FITC (FL1)	Invitrogen
	Mo	Go	Poly	$F(ab')_2$	-	AF488 (FL1)	Invitrogen
Negative	-	Mo	Mono	IgG2a	UPC-10	-	Sigma
control	-	Mo	Mono	IgG2b	MOPC-141	-	Sigma
	-	Mo	Mono	IgG1	MOPC-21	-	In house
	-	Mo	Mono	IgG1	3	FITC (FL1)	AbD Serotec
	-	Mo	Mono	IgG1	3	R-PE (FL2)	AbD Serotec
P-tyrosine	-	Mo	Mono	IgG2b	PY-20	Biotin	BD Biosciences
Biotin	-	Mo	Mono	IgG1	BN-34	FITC (FL1)	Sigma

Notes: ***1***. Provided from Ian Dransfield, University of Edinburgh; ***2***. Gift from Martin J. Glennie, University of Southampton.

Abbreviations: Ig: immunoglobulin; P: phospho; Hu: human; Ha: hamster; Mo: mouse; Go: goat; Ra: rabbit; Mono: monoclonal; Poly: polyclonal; FITC: Fluorescein isothiocyanate; R-PE: R-Phycoerythrin; HRP: horseradish peroxidase; AF: AlexaFluor™.

11.2. Bacterial Strains

Bacterial strains used in this study were the following *E.coli* K12 derivatives: JM109 (*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′ *tra*D36, *pro*AB, *laq*I^{q}ZΔM15]) obtained from Promega and DH5α (F$^-$ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-) from Invitrogen.

Unless otherwise stated, bacterial cells were grown in the complex LB media (1% w/v tryptone, 0.5% w/v yeast extract, 85 mM NaCl; Sigma Aldrich). For long term storage, bacterial glycerol stocks (10% v/v glycerol in Terrific Broth (TB, Sigma Aldrich; 1.2% tryptone, 2.4% yeast extract, 54 mM K_2HPO_4, 16 mM KH_2PO_4, 0.4% v/v glycerol) were prepared and stored at -80°C.

11.3. Plasmid Constructs

The following plasmids were used in this study:

pJ201:CD55-GPI: ampicillin resistant, pJ201 vector (DNA2.0 Inc., Menlo Park, California, USA) containing the GPI-modification consensus sequence flanked by *Xho*I and *Bln*I restriction sites at the 5' and 3' end, respectively. This construct contained no mammalian selection marker.

p-SELECT-neo-mcs-FCGR2A: kanamycin/neomycin resistant, p-SELECT-based vector (Invivogen, Autogen Bioclear, Calne, Wiltshire, UK) with the *FCGR2A* ORF cloned at the multiple cloning site (mcs). Protein was expressed under the control of a hEF1/HTLV mammalian promoter. This plasmid construct was

used as the template for all site-directed mutagenesis reactions. The following derivatives of the p-SELECT-neo-mcs-*FCGR2A* were created in this study: (i) p-SELECT-neo-mcs-*FCGR2A* A224S, and (ii) p-SELECT-neo-mcs-*FCGR2A* C241A containing the A224S and C241A mutation within the *FCGR2A* ORF, (iii) p-SELECT-neo-mcs-*FCGR2A* sh78mm, containing the original protein sequence of CD32a, but with incorporated silent mutations within the shRNA-recognising sequence of clone TRCN029578 (recognising sequence 5'-GAAGAAACCAACAATGACTAT) to prevent targeting by the shRNA, (iv) p-SELECT-neo-mcs-*FCGR2A Xho*I, engineered with an *Xho*I restriction site at the extracellular, membrane proximal region of CD32a (from the pSELECT-noe-mcs-*FCGR2A* sh78mm vector) used for the generation of CD32/CD55 chimaeric protein, and (v) p-SELECT-neo-mcs-*FCGR2A/CD55*-GPI, expressing the chimaeric CD32a/CD55 protein (GPI-anchored CD32a) instead of CD32a.

Mission shRNA clones were obtained from Sigma Aldrich and were based on the pLKO.1-puro vector system, carrying ampicillin and puromycin resistance genes. shRNA-expressing sequences were constitutively under the control of human polymerase III (Pol III) U6 promoter. These shRNA clones were the following: TRCN029574 (target sequence on CD32a: 5'-GCCATCAGAAAGAGACAACTT, TRCN029575 (5'-GCACCTACTGACGATGATAAA), TRCN029576 (5'-CCAGAAATTCTCCCGTTTGGA), TRCN029577 (5'-CCATGTCAACAGTAATAACTA), TRCN029578 (5'-GAAGAAACCAACAATGACTAT). All the plasmid maps and sequences are presented in *Section 15.1.*

11.4. Clinical Study Subjects

All subjects in the clinical study were of Caucasian origin and provided written informed consent. Ethical approval was obtained from the Lothian Research Ethics Committee (LREC/2002/4/65) and from the Lothian University Hospital – National Health Service (NHS) trust (R&D department) (2002/R/UO/22). Control patients (n=218) included healthy blood donors and patients admitted at the Respiratory Medicine Unit of the Royal Infirmary of Edinburgh with minor and non-chronic lung pathologies, without any evidence or history of lung fibrosis. The diagnosis of IPF was made based on the ATS/ERS international multidisciplinary consensus classification (ATS/ERS, 2002), according to the following criteria: (i) exclusion of all known causes or associations with lung fibrosis, including drug toxicities, connective tissue disease or exposure to environmental agents, (ii) presence of typical features on high resolution computed tomography scans, including bibasilar lung honeycombing with minimal ground glass opacities, (iii) abnormal pulmonary function with evidence of restriction (reduced FVC (forced expiratory vital capacity), often with an increased FEV_1(forced expiratory volume in 1 second)/VC (vital capacity) ratio) and/or reduced gas transfer measurements (decreased D_{LCO} (diffusing capacity for carbon monoxide)), (iv) age >50 years and (v) duration of illness >3 months (*Table 7.2*). When bronchoalveolar lavage (BAL) or transbronchial lung biopsy was performed, no features were evident to support an alternative diagnosis. Surgical lung biopsy and/or BAL were performed in cases for which a confident diagnosis on clinical, functional

and radiological grounds was not possible. A consensus diagnosis was made in each case following joint review by two respiratory clinicians and a radiologist (and a pathologist for cases in which biopsy was performed). In 39 of 142 patients the diagnosis was confirmed by surgical biopsy, which revealed a histological profile typical of usual interstitial pneumonia. Pulmonary function measurements were recorded at baseline (first radiologic evidence for IPF; date of first HRCT scan) and included FEV_1, FVC, TLC (total lung capacity) assessed by spirometry. In addition, the diffusing capacity for carbon monoxide (D_{LCO}) and K_{CO} (corrected D_{LCO} for alveolar volume) were measured by the single breath technique. FVC and D_{LCO} were monitored for at least 12 months following disease diagnosis (baseline) to assess disease progression and prognosis in 121 IPF patients. For the remaining 21 patients, we were unable to obtain serial lung function measurements, as either contact with the patients was lost or they were unfit to participate in multiple pulmonary function tests.

11.5. Cell Isolation and Culture

11.5.1. Cell Lines and Culture

The following cell lines were used in this study: K562 cells (ATCC No.: CCL-243), a human erythromyeloid leukaemic cell line, CHO-K1 cell line (CCL-61), a derivative of the Chinese hamster ovary (CHO) cell line, with epithelial-like morphology, HL-60 cell line (CCL-240), a human promyelocytic cell line, mouse monoclonal (IV.3) antibody-producing hybridoma cells

(HB-217) and Jurkat cells that were previously stably transfected to express human CD16 and or CD32 (described in Green *et al.* (1997)). In brief, the sham-transfected Jurkat cell line was transfected with the empty pRc/ CMV:FLAG vector, CD32-expressing cells with the pRc/CMV:CD32a vector and CD16-expressing cells with the pCEP4:CD16b vector. CD16- and CD32-expressing Jurkat cells were co-transfected with both the pRc/CMV:CD32a and the pCEP4:CD16b vectors.

Unless otherwise stated, K562 and Jurkat cells were cultured in RPMI 1640 medium (PAA or Invitrogen) supplemented with 10% foetal calf serum (FCS), L-glutamine (2 mM) and penicillin (100 U ml^{-1})/streptomycin (100 µg ml^{-1}). HL-60 cells were cultured in RPMI 1640 medium (PAA or Invitrogen) supplemented with 20% foetal calf serum (FCS), L-glutamine (2 mM) and penicillin (100 U ml^{-1})/streptomycin (100 µg ml^{-1}). K562, Jurkat and HL-60 cells were maintained at a cell density of $1x10^5$-$5x10^5$ cells ml^{-1}. Chinese Hamster Ovary (CHO-K1) cells were maintained in D-MEM:F-12 (1:1) (Gibco, Invitrogen) with GlutaMAX™, 10% FCS and penicillin (100 U ml^{-1})/ streptomycin (100 µg ml^{-1}). IV.3 hybridoma cells were cultured in IMDM supplemented with 10% heat-inactivated FCS, 4 mM L-glutamine, 25 mM HEPES, penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}) and maintained at a density of 1-$2x10^6$ cells ml^{-1}. All cells were incubated at 37°C, 5% CO_2 in a humidified atmosphere.

11.5.2. Leukocyte Isolation

Ethical approval was obtained from the Lothian Local Research Ethics Committee (approval 1702/95/3111 and 08/S1103/38) and all human subjects provided oral or written informed consent, as required. Peripheral venous blood from healthy volunteers was drawn via a 19-gauge needle from an antecubital vein and collected into polypropylene tubes containing sodium citrate as an anticoagulant (12.9 mM final concentration). Citrated blood was then immediately processed for leukocyte isolation, based on previously described protocols (Bournazos *et al.*, 2008; Dransfield *et al.*, 1994).

Briefly, following centrifugation of whole blood at 350*g* for 20 min, platelet-rich plasma was removed and used for platelet isolation and the generation of autologous serum (see below). Erythrocytes were sedimented from the cell-enriched layer by dilution 1:1 with sterile saline pre-warmed to 37°C with the addition of dextran T-500 (Pharmacia, GE Healthcare) solution (prepared in sterile saline solution) at a final concentration of 0.6% (w/v). The leukocyte-rich layer was then washed once with saline solution (350*g*; 6 min) and mononuclear leukocytes were fractionated from polymorphonuclear leukocytes by centrifugation at 720*g* for 20 minutes using discontinuous isotonic Percoll™ gradients (final Percoll™ concentration: 50%, 61%, 73%; GE Healthcare). Mononuclear cells were harvested from the 61%/50% interface and polymorphonuclear cells from the 73%/61%. Harvested cells were washed twice with phosphate buffered saline (PBS) (Ca^{2+}, Mg^{2+} free) and

purity was routinely assessed by flow cytometry and/or microscopic examination of Diff-Quick-stained cytocentrifuge preparations.

Monocytes ($CD14^+$) represented 8-25% of total mononuclear cell population and neutrophils ($CD16^+$) comprised ≥95% of the polymorphonuclear cell fraction. Cell viability was routinely assessed by Trypan blue exclusion and cells were ≥99% viable. Unless otherwise stated, isolated cells were used immediately for subsequent experiments. Autologous serum was prepared by re-calcification of platelet-rich plasma (final $CaCl_2$ concentration: 22 mM) and incubation in sterile glass tubes at 37°C for at least 1 h. Serum was stored for future use at 4°C.

11.5.3. Generation of Human Monocyte-Derived Macrophages

Freshly isolated mononuclear cells (described in *Section 11.5.2*) were re-suspended in IMDM at $4x10^6$ cells ml^{-1} and allowed to adhere to 48-well tissue culture plates (Corning-Costar, Fisher Scientific UK, Loughborough, Leicestershire, UK) for 1 h at 37°C (5% CO_2). Non-adherent lymphocytes were removed by extensive washing with Hanks' Balanced Salt Solution (HBSS) (containing Ca^{2+}/Mg^{2+}) and adherent monocytes were cultured for 6 days in IMDM containing 4 mM L-glutamine, penicillin (100 U ml^{-1})/streptomycin (100 μg ml^{-1})(PAA Laboratories) and supplemented with 10% autologous serum at 37°C (5% CO_2). Culture medium was replaced every three days.

Cells displaying macrophage-specific functional and phenotypic characteristics, as assessed by morphologic criteria and flow cytometric analysis represented >80% of the total population. For immunolabelling and analysis by flow cytometry, macrophages were washed once with HBSS (Ca^{2+}/ Mg^{2+} free) and incubated with pre-warmed HBSS containing 5 mM EDTA, 5 mM sodium citrate and 0.5% FCS for 15 min at 37°C, followed by 15 min on ice. Cells were then harvested from the tissue culture plate by forceful pipetting. For use for the phagocytosis assay, cells were detached following phagocytosis with PBS containing 0.05% (w/v) Trypsin and 0.02% EDTA to remove adherent, non-ingested apoptotic cells from the macrophage surface, as described *Section 11.12*.

11.6. Immunolabelling and Flow Cytometry

For the determination of cell surface molecule expression, cells were directly or indirectly immunolabelled and examined by flow cytometry. For direct immunolabelling, cells were washed once in PBS and re-suspended at a final concentration of $5x10^6$ cells ml^{-1} in ice-cold PBS containing 1% FCS and the corresponding fluorochrome-conjugated antibody or isotype control antibody was added at saturating concentration (as determined by titration analysis). Cells were incubated on ice protected from light for 20 min and then, washed once with ice-cold PBS (1% FCS), prior to flow cytometric analysis. For indirect immunolabelling, cells were incubated with the primary antibody (10 $\mu g\ ml^{-1}$, diluted with PBS containing 1% FCS) for 30 min on ice. Then, cells

were washed twice with ice-cold PBS (1% FCS) and incubated for 20 min on ice with the corresponding fluorochrome-conjugated secondary antibody (10 μg ml^{-1}) to detect primary antibody binding. Unless otherwise stated, secondary antibodies were $F(ab')_2$ fragments to minimise cross-reactivity with Fcγ receptors. Following incubation with the secondary antibodies, cells were washed twice with ice-cold PBS (1% FCS) and analysed immediately by flow cytometry. Flow cytometric analysis of the samples was performed using a BD FACS Calibur® or FACScan® cytometer (BD Biosciences). Data were analysed using BD CellQuest™ (BD Biosciences), BD FACS Diva® (BD Biosciences) or FlowJo™ (TreeStar™, Ashland, Oregon, USA) software and unless otherwise stated, results are presented as the geometric mean fluorescence intensity.

11.7. IV.3 (anti-CD32a) Antibody Purification

IV.3 hybridoma cells (mouse $IgG2_b$, κ) were cultured as described in *Section 11.5.1* and cell-free supernatants were collected. Supernatants were filtered with 0.22 μm pore filters, pH was adjusted to 8.0 and stored at 4°C until used for antibody purification. An aliquot of the supernatant was used for indirect immunolabelling of CD32a-transfected CHO cells, to confirm the presence of functional antibody in the collected supernatant. Antibody was purified using a 4 ml Protein G-Sepharose™ (GE Healthcare) column and all procedures were performed at 4°C. The column was equilibrated with 50 ml of 0.1 M phosphate buffer (pH 8.0) at a rate of 5 ml min^{-1}. Supernatants were then slowly applied to the column at a rate of 0.5 ml min^{-1}. The column was then washed with 15

ml of 0.1 M citrate buffer, pH 6.0, collecting 1 ml fractions and the protein content was estimated by UV spectrophotometry at 280 nm. Column was washed with 0.1 M citrate buffer, pH 6.0 until A_{280} was essentially zero. Bound antibody was eluted from the Protein G column, following wash with 0.1 M citrate buffer, pH 3.0. Fractions (1 ml) were collected and pH was immediately adjusted by the addition of 1 M Tris, pH 9.0. A_{280} was measured in the collected fractions and antibody-containing fractions identified by measurement of absorbance at 280 nm were pooled and concentrated using an Amicon® concentrator device (10,000 Da molecular weight cut-off filter, Amicon®, Millipore, Watford, Hertfordshire, UK) under helium pressure. Purified antibody was then dialysed against PBS (Ca^{2+}/Mg^{2+}-free), sodium azide (Sigma Aldrich) was added to a final concentration of 0.1% (w/v) and stored at 4°C. The specificity and binding of the purified antibody was assessed by indirect immunolabelling of CD32-expressing CHO cells and/or isolated neutrophils (protocol described in *Section 11.6*) and antibody titre determined.

Antibody purification from IV.3 hybridoma supernatants was also performed with slight modifications to the above-described protocol. In detail, cell-free supernatants (0.22 μm filtered and pH adjusted to 8.0) were used for ammonium sulfate precipitation, as previously described by Harlow and Lane (1988), to increase antibody concentration of the supernatant. Saturated ammonium sulfate solution (4.1 M, pH 7.5) was slowly added drop-wise to an equal volume to hybridoma supernatant, which was continuously and vigorously stirred to ensure adequate mixing. The mixture was incubated at

4°C without stirring for 20 h to allow antibodies to precipitate and transferred to 50 ml polypropylene tubes. Precipitated material was then pelleted by centrifugation in a swinging-bucket rotor (3700*g* for 30 min) and re-suspended by slowly adding minimal volume of PBS (Ca^{2+}/Mg^{2+} free), until completely dissolved. Solution was then dialysed against 0.1 M phosphate buffer (pH 8.0) and applied to the Protein G-Sepharose column, following the protocol described above.

11.8. Protein Biotinylation

Purified proteins were biotinylated using the EZ-Link™ Sulfo-NHS-LC-Biotin reagent (Pierce/ Thermo Fisher Scientific), according to manufacturer's instructions. Purified protein (BSA or IgG) was suspended in PBS, unless otherwise stated and incubated with freshly prepared biotinylation reagent for 2 h at room temperature. EZ-Link™ Sulfo-NHS-LC-Biotin was added at a final molar ratio of 20:1 (biotin reagent:protein). Biotinylated protein was then dialysed against PBS (Ca^{2+}/Mg^{2+} free) (4°C) using Slide-A-Lyzer® dialysis cassettes (Pierce/ Thermo Fisher Scientific) with 10,000 Da molecular weight-cut-off (MWCO) membranes, according to manufacturer's recommendations.

11.9. CD32 Immunoprecipitation and Immunoblotting

Cells were re-suspended ($2x10^7$) in PBS (Ca^{2+}/Mg^{2+} free) and surface-biotinylated by incubation with biotin (EZ-Link™ Sulfo-NHS-LC-Biotin; 1 mg ml^{-1}) for 60 min on ice. Then, cells were washed extensively with PBS (Ca^{2+}/

Mg^{2+} free) containing 20 mM Tris (pH 8.0) to remove non-reacted biotin. Cells were lysed at a density of $2x10^7$ cells ml^{-1} with 1% (v/v) NP-40 (nonyl phenoxylpolyethoxylethanol) in TBS (Tris Buffered Saline, 50 mM Tris, 150 mM NaCl; pH 7.5) containing protease inhibitors (Complete mini protease inhibitor tablets, Roche Applied Sciences). Following a 30-min incubation on ice, lysates were cleared by centrifugation (14,000*g*; 15 min, 4°C). Samples (250 µl) were then incubated with agarose-conjugated anti-CD32 antibody (50 µl, clone IV.3) for 2 h at 4°C.

Agarose pellets were then extensively washed (12 times) using ice-cold lysis buffer and proteins were resolved by SDS-PAGE using 9% or 5-20% gradient Tris-glycine gels (ePAGEL, Atto Corporation). Proteins were electrophoretically transferred onto nitrocellulose membrane (Amersham Biosciences) for 40 min at 100 V in 25 mM Tris, 192 mM Glycine pH 8.3 containing 20% methanol and blocked with 0.1% (v/v) Tween®-20. Blots were probed with HRP-conjugated streptavidin (1:5000) and visualised using enhanced chemiluminescence (ECL™ reagent; Amersham Biosciences) and detection using ECL hyperfilm™ (Amersham Biosciences; GE Healthcare).

For CD32 immunoblotting, cells were washed twice with ice-cold PBS (Ca^{2+}/ Mg^{2+} free) and incubated with 1 mM PMSF for 30 min on ice. Cells were then lysed with 1% (v/v) NP-40 in TBS containing protease inhibitors (Complete mini protease inhibitor tablets, Roche Applied Sciences) at a final density of $1.5x10^7$ cells ml^{-1} and incubated on ice for 30 min prior to centrifugation (14,000*g*, 15 min, 4°C). Proteins were immediately resolved by SDS-PAGE

using 9% or 12% Tris-glycine gels and electrophoretically transferred onto nitrocellulose membrane (Amersham Biosciences) as described above. Membranes were blocked with 5% (w/v) fat-free milk, then probed with goat polyclonal anti-CD32 (1:500; R&D systems), followed by HRP-conjugated rabbit anti-goat Ig (1:5000; Dako Cytomation) and visualised using enhanced chemiluminescence (ECL™ reagent; Amersham Biosciences) and detection using ECL hyperfilm™ (Amersham Biosciences; GE Healthcare).

11.10. Immune (IgG) Complex Binding Assay

11.10.1. Generation of Immune Complexes

Immune complexes were generated as previously described (Hart *et al.*, 2004a). Briefly, for biotin – anti-biotin complexes (BxB), FITC-conjugated anti-biotin (mouse IgG1, clone BN-34; 170 μg ml^{-1}) was co-incubated with biotin-conjugated bovine serum albumin (500 μg ml^{-1}) at 4°C for 30 min. For the generation of fetuin – anti-fetuin complexes (Bob93) serum-free supernatants from anti-fetuin-producing hybridoma cells (clone Bob93, mouse IgG1) were co-incubated with FITC-conjugated purified fetuin (0.5 mg ml^{-1}) at 4°C for 30 min (Hart *et al.*, 2003). Human IgG complexes (human heat-aggregated IgG; hHAIgG) were formed by incubating monomeric human IgG (10 mg ml^{-1}) for 20 min at 63°C and centrifuged at 14,000*g* to remove protein precipitates. For human heat-aggregated IgG complexes, either FITC-conjugated or unconjugated IgG was used. In all cases, complexes were found to be stable when stored for at least one month at 4°C undiluted.

11.10.2. Measurement of Immune Complex Binding

Immune (IgG) complex binding was assessed based on a previously described assay that measures binding of fluorochrome conjugated- IgG complexes to CD32 by flow cytometry (Hart *et al.*, 2004a; Hart *et al.*, 2003). Cells were washed once in PBS (Ca^{2+}/Mg^{2+} free) and re-suspended in ice-cold PBS containing 0.1% (w/v) IgG-free BSA (Sigma Aldrich) at a final density of 10^6 cells ml^{-1}. Cells were incubated with varying concentrations of IgG complexes (diluted in PBS with 0.1% (w/v) BSA) for 30 min on ice, then washed twice with PBS containing 0.1% (w/v) BSA and analysed immediately by flow cytometry. For blocking experiments, function blocking anti-CD32 antibodies (IV.3 or AT-10 $F(ab')_2$; 10 μg ml^{-1}) or anti-CD16 (3G8 $F(ab')_2$; 10 μg ml^{-1}) were added 10 min prior to the addition of IgG complexes. Flow cytometric analysis of the samples was performed using a BD FACS Calibur™ or FACScan™ cytometer (BD Biosciences). Data were analysed using BD CellQuest™ (BD Biosciences), BD FACS Diva™ (BD Biosciences) or FlowJo™ (TreeStar) software and all results are presented as the median fluorescence intensity.

11.11. Induction and Assessment of Neutrophil Apoptosis

Unless otherwise stated, freshly isolated neutrophils were cultured at $4x10^6$ cells ml^{-1} in IMDM containing 4 mM L-glutamine, penicillin (100 U ml^{-1})/ streptomycin (100 μg ml^{-1})(PAA Laboratories) and supplemented with 10% heat-inactivated foetal bovine serum (FBS) at 37°C for 20 h (5% CO_2), during which time a proportion of the cells underwent spontaneous apoptosis.

Neutrophil apoptosis and secondary necrosis were assessed by annexin-V binding and propidium iodide staining, respectively. In brief, following overnight culture, neutrophils were re-suspended in annexin binding buffer (HBSS (Mg^{2+} free) containing 5 mM $CaCl_2$) at a final concentration of $4x10^6$ cells ml^{-1}. APC-or FITC-conjugated recombinant annexin-V (Caltag, Invitrogen) was then added (diluted 1:50 in annexin binding buffer) to the cells and incubated for 10 min on ice. Propidium iodide (Sigma Aldrich) was added immediately prior to flow cytometry analysis at a final concentration of 1 μg ml^{-1}. Cells were then analysed by flow cytometry using a BD FACS Calibur™ flow cytometer (BD Biosciences) and data were analysed using BD CellQuest™ (BD Biosciences), BD FACS Diva™ (BD Biosciences) or FlowJo™ (TreeStar) software.

11.12. Macrophage Phagocytosis Assay

11.12.1. Cell Labelling for Phagocytosis Assay

Cells were labelled with either one of the following cell-permeable, long-term dyes: 5-chloromethylfluorescein diacetate (CMFDA; CellTracker™ Green, Molecular Probes, Invitrogen), or 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE)(CellTrace™ Far Red, Molecular Probes, Invitrogen), detected in FL-1 and FL-4 channels in flow cytometry, respectively. These reagents were prepared in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM, protected from light and the following protocol was used for cell labelling (Hart *et al.*, 2008). Freshly

isolated neutrophils were suspended in IMDM, containing 4 mM L-glutamine, 25 mM HEPES, penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}) at a final density of 10^7 cells ml^{-1} and cells were incubated with cell labelling reagent (final concentration of 20 µM) at 37°C (5% CO_2) for 15 min. Cells were then washed once, re-suspended in IMDM (4 mM L-glutamine, 25 mM HEPES, penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1})) supplemented with 10% FCS and cultured at 37°C (5% CO_2) for 20 h, as described in *Section 11.11*. The extent of neutrophil apoptosis was determined in unlabelled cells that were cultured in identical conditions.

11.12.2. Flow Cytometry-based Apoptotic Cell Phagocytosis Assay

Macrophage phagocytosis of apoptotic cells was assessed as previously described, using a method that measures macrophage-mediated internalisation of fluorescently labelled apoptotic cells by flow cytometry (Hart *et al.*, 2008; Jersmann *et al.*, 2003). Following labelling and *in vitro* culture of freshly isolated neutrophils for 20 h at 37°C (5% CO_2)(described in *Sections 11.11 and 11.12.1*), cells were washed once in PBS (Ca^{2+}/Mg^{2+} free) and were incubated (10^7 cells ml^{-1}) for 20 min on ice with either PBS or heat-aggregated human IgG (100 µg ml^{-1})(prepared as described in *Section 11.10.1*). Cells were then washed once with PBS (Ca^{2+}/Mg^{2+} free) and re-suspended at $2x10^6$ cells ml^{-1} in pre-warmed IMDM containing 25 mM HEPES and 4 mM L-glutamine without serum. Culture medium was removed and the macrophage monolayer washed once with pre-warmed medium (IMDM without serum) to remove non-adherent cells (contaminating lymphocytes and dead cells).

For experiments involving the block of macrophage Fcγ receptors, macrophages were incubated with saturating concentrations of the relevant monoclonal antibody (10 μg ml^{-1}), diluted in PBS containing 1% (v/v) of autologous serum) and incubated for 15 min at 37°C (Vivers *et al.*, 2004). Medium was then aspirated and for each well of a 48-well culture plate, $1x10^6$ "aged" neutrophils were added ($2x10^6$ cells ml^{-1}) and incubated for 60 min at 37°C (5% CO_2). For experiments involving the use of cell populations labelled with different dyes, cells were added at 1:1 ratio (CMFDA-labelled:DDAO-SE-labelled). Following incubation, medium was aspirated and cells were detached with 0.5 ml of PBS containing trypsin (0.05% (w/v))/EDTA (0.02% (w/v)) solution. Cells were incubated with the detaching solution for 10 min at 37°C, followed by 10 min at 4°C. Then, FBS was added to the cells at a final concentration of 0.5% (v/v) to competitively inhibit trypsin activity and limit cell aggregation and detached cells were harvested by forceful pipetting. Samples were analysed by flow cytometry using a BD FACS Calibur™ flow cytometer (BD Biosciences) and at least 5,000 events in the macrophage gate were collected. Apoptotic cells and macrophages were identified based on their characteristic autofluorescence, forward and side scatter laser properties. In particular, when neutrophils were labelled with the far red dye DDAO-SE (detected in FL-4 channel), the macrophage population was identified on the basis of increased FL-1 autofluorescence and greater forward scatter laser properties compared with the other cell types (apoptotic neutrophils, contaminating lymphocytes and erythrocytes) present. The FL-4^{bright} and FL-4^{dim} macrophages represented the phagocytic and non-

phagocytic macrophage populations, respectively. Similarly, in phagocytosis assays using CMFDA-labelled apoptotic neutrophils, FL-1^{bright} macrophages defined on the basis of forward and side scatter laser properties represented those with internalised apoptotic cells. Data were analysed using BD CellQuest™ (BD Biosciences), BD FACS Diva™ (BD Biosciences) or FlowJo™ (TreeStar) software and unless otherwise stated, results are presented as the percentage of FL-1^{bright} or FL-4^{bright} (depending on the fluorescent dye used) events in the macrophage gate from the total number of macrophages. For the determination of the flow cytometric phagocytic index, the percentage of phagocytic macrophages (FL-4^{bright} or FL-1^{bright}) was multiplied by the geometric mean fluorescence (either FL-4 or FL-1) of the phagocytic population.

11.12.3. Microscopy-based Apoptotic Cell Phagocytosis Assay

Microscopy-based assessment of phagocytosis was performed essentially the same as the flow cytometry-based assay, with the exception that apoptotic neutrophils were not labelled prior to use in the assay. Instead, myeloperoxidase (MPO) activity that is exclusively found in neutrophil granules was detected using a colourimetric method (Hart *et al.*, 2004a; Hart *et al.*, 2008). Macrophages and neutrophils were cultured and prepared for the phagocytosis assay as described for the flow cytometry-based phagocytosis assay. Following co-incubation of macrophages with apoptotic neutrophils (60 min, 37°C, 5% CO_2), macrophage cell monolayer was extensively washed with ice-cold HBSS (containing Ca^{2+}/Mg^{2+}) to remove non-ingested apoptotic

neutrophils. To ensure complete removal of non-ingested apoptotic cells, macrophages were inspected by light microscopy. Cells were fixed with 3% (w/v) paraformaldehyde for 30 min at room temperature and cell monolayer was rinsed once prior to staining for MPO. Cells were incubated with 0.1 mg ml^{-1} 3,3'-dimethoxybenzidine (Sigma Aldrich) containing 0.03% (v/v) hydrogen peroxide for approximately 60 min or until ingested neutrophils developed a brown colour, while macrophages remained unstained. Cells were rinsed with PBS (Ca^{2+}/Mg^{2+} free) and stored at 4°C in PBS containing 0.1% (w/v) sodium azide until further analysis. For the determination of macrophage phagocytosis, the proportion of macrophages (MPO negative) that had ingested one or more apoptotic neutrophils (MPO positive) was quantified (at least 300 cells per well) using an inverted microscope. In addition, the phagocytic index was determined as the number of ingested apoptotic neutrophils present in 100 macrophages. Unless otherwise stated, results are expressed as the mean (either percent phagocytosis or phagocytic index) for at least three experiments performed in duplicate wells.

11.13. Cytokine Measurement

Cell-free supernatants from LPS-treated (50 ng ml^{-1}, 18 h, 37°C) macrophages were collected by centrifugation (14,000*g*, 2 min) and stored at -20°C until analysis. Cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α were measured in these samples using a fluorescent bead-based sandwich assay (cytometric bead array (CBA); human inflammation kit, BD Biosciences),

according to manufacturer's instructions. Briefly, 25 µl of cytokine standard or cell-free supernatant were co-incubated with 25 µl of capture beads (mixed at equal volumes for each cytokine) and 25 µl secondary PE-conjugated detection reagent for 3 h at room temperature protected from light. Beads were then washed with the CBA wash buffer (200*g*, 5 min) and bead pellet was carefully vortexed and re-suspended in CBA wash buffer. Analysis of the samples was performed using a BD FACS Array™ Bioanalysis System (BD Biosciences), using FL-2 and FL-3 detection channels, based on pre-installed settings for the Human Inflammation kit. Standard curves were calculated and data were analysed using BD CBA Software (BD Biosciences).

11.14. Measurement of NADPH Oxidase Activity

11.14.1. Measurement of Intracellular ROS levels

Intracellular ROS levels were determined in neutrophils using a fluorescent probe, dihydrorhodamine123 (DHR123), which upon reacting with intracellular ROS (mainly superoxide and H_2O_2), is oxidised to rhodamine 123 that is excitable at 488 nm and is emitted at a wavelength of 515 nm. Freshly isolated neutrophils (control or protease-treated) were re-suspended at a final concentration of $2.5x10^6$ cells ml^{-1} in pre-warmed HBSS containing Ca^{2+}/Mg^{2+}. Cells were incubated for 5 min at 37°C with 1 µM DHR123 dye and then, human heat aggregated IgG complexes (0.1-100 µg ml^{-1}) or fMLP (100 nM) were added and cells were further incubated for 60 min at 37°C. Cells were then immediately transferred on ice and analysed by flow cytometry using a

BD FACS Calibur™ or BD FACScan™ (BD Biosciences) flow cytometer and fluorescence was detected using the FL-1 detection channel. Data were analysed using BD CellQuest™ (BD Biosciences), or FlowJo™ (TreeStar) software. Background fluorescence (baseline ROS levels) was subtracted from each corresponding sample and results are presented as the median fluorescence intensity of at least three independent experiments.

11.14.2. Superoxide Release Assay

The generation and release of superoxide from stimulated neutrophils was measured, essentially as previously described, based on cytochrome c reduction (Stocks *et al.*, 1995). Briefly, neutrophils ($5x10^5$ cells ml^{-1}) were suspended in HBSS (with Ca^{2+}/Mg^{2+}) containing cytochrome c (1 mg ml^{-1}; Sigma Aldrich) in the presence of an agonist (PMA: 100 nM or human heat aggregated IgG: 0-200 µg ml^{-1}). Samples were incubated at 37°C for 45 min and then cells were immediately transferred on ice. Supernatants were obtained by centrifugation (300*g*, 5 min) and absorbance was measured at 550 nm using a BioTek™ microplate UV spectrophotometer (BioTek Instruments, Potton, Bedfordshire, UK). Spontaneous cytochrome c oxidation or background autofluorescence was subtracted from the non-cell sample and results are presented as the mean absorbance of three independent experiments performed in triplicates.

11.15. Analysis of CD32 Tyrosine Phosphorylation

Following stimulation of CHO cells with IgG complexes (10 μg ml^{-1}, 5 min, 37°C), cells ($3x10^7$ cells ml^{-1}) were washed extensively in ice-cold PBS and lysed with 2% Triton X-100 in Tris buffered saline (150 mM NaCl, 50 mM Tris, pH 7.5), in the presence of phosphatase and protease inhibitors (Halt™ Protease and Phosphatase Inhibitor Cocktail, Pierce/Thermo Scientific) for 15 min on ice, based on manufacturer's recommendations. Lysates were then cleared of insoluble material by centrifugation (14,000*g*, 15 min, 4°C) and incubated with agarose-conjugated goat anti-mouse Ig (Sigma Aldrich) for 60 min at 4°C to minimise non-specific protein interactions. Anti-CD32 antibody (IV.3; 1 μg per 0.25 ml sample) was then added to the pre-cleared lysates and incubated for 60 min at 4°C, followed by goat anti-mouse Ig agarose (25 μl) for 90 min at 4°C shaking. Agarose pellets were then extensively washed (12 times) using ice-cold lysis buffer and proteins were resolved by SDS-PAGE using 4-12% Bis-Tris gels (NuPAGE, Invitrogen), according to manufacturer's instructions. Proteins were then electroblotted onto nitrocellulose membrane (Amersham), blocked with 0.1% (v/v) Tween™-20 and probed with biotin-conjugated mouse anti-phosphotyrosine (1:7000). Membranes were then incubated with HRP-conjugated streptavidin (1:5000) and visualised using enhanced chemiluminescence (ECL™ reagent; Amersham Biosciences). To assess total CD32 content, membranes were stripped with 0.1 M glycine pH 2.5 containing 0.1% (v/v) Tween™-20 (45 min at room for temperature) and then blocked with 5% w/v fat-free milk in PBS Tween™-20 (0.1% v/v) for 60 min.

Blots were then re-probed with goat polyclonal anti-human CD32a (1:500), followed by HRP-conjugated rabbit anti-goat Ig (1:5000).

11.16. Nucleic Acid Purification

11.16.1. Molecular Biology-grade Bacterial Plasmid DNA Extraction

Plasmid DNA extraction was performed using the Qiagen QIAprep™ Spin MiniPrep Kit (Qiagen), according to manufacturer's recommendations. Briefly, under aseptic conditions, a single *E.coli* colony was inoculated to pre-warmed (37°C) LB medium (10 ml) containing the corresponding selection antibiotic (kanamycin (50 μg ml^{-1}) or carbenicillin (100 μg ml^{-1}) and cultures were incubated overnight at 37°C shaking (250 rpm). Overnight cultures (10 ml) were then centrifuged (3700*g*, 10 min) and bacterial pellets were re-suspended in Buffer P1 (250 μl), containing LyseBlue™ reagent and RNase A (100 μg ml^{-1}). Bacterial cells were lysed following the addition of Buffer P2 (250 μl), mixed thoroughly by gentle inversion (4-6 times). Lysates were neutralised immediately by adding 350 μl of Buffer N3. Following careful and thorough mixing, lysates were cleared of protein precipitates by centrifugation (10 min, 14,000*g*) and supernatants were applied to the QIAprep™ spin column. The column was centrifuged (14,000*g*, 1 min) and washed by the addition of buffer PB (0.75 ml) and further centrifuged for 1 min (14,000*g*). Column flow-though was discarded and the QIAprep™ Spin column was again washed using Buffer PE (0.75 ml). After a 1-minute centrifugation at 14,000*g*, flow-through was discarded and the column was further centrifuged for an

additional 1 min to completely remove residual wash buffer. Plasmid DNA was then eluted from the column by adding 50 µl Tris (10 mM), pH 8.5, incubated for 1 min at room temperature and centrifuged (14,000*g*, 1 min). Flow-through containing the eluted plasmid was stored at -20°C. Plasmid concentration was determined by spectrophotometric analysis over a 230-300 nm absorbance range using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) and absorbance 260 nm/280 nm ($A_{260/280}$) ratio values between 1.8 and 2.0 were considered to be optimal and indicative of pure DNA preparations. The quality of the extracted plasmid DNA was also assessed by agarose (1% w/v) gel electrophoresis.

11.16.2. Transfection-grade Bacterial Plasmid DNA Extraction

For the extraction and purification of plasmid DNA intended for cell transfection purposes, the Qiagen Plasmid Maxi kit was used essentially based on manufacturer's guidelines. In detail, single *E.coli* colonies were inoculated to pre-warmed (37°C) LB medium (5 ml) containing the relevant plasmid selection antibiotic (kanamycin (50 µg ml^{-1}) or carbenicillin (100 µg ml^{-1})). Following an 8-hour incubation period (37°C; 7-8 h, 250 rpm), starter cultures were diluted into selective LB medium (1 ml of the culture was added to 500 ml LB medium) and further incubated for 16 h at 37°C (250 rpm). Bacterial cells were harvested by centrifugation (6,000*g*, 15 min, 4°C) and bacterial pellets were thoroughly re-suspended in 10 ml of ice-cold buffer P1, containing LyseBlue™ reagent and RNase A (100 µg ml^{-1}). Lysis buffer (Buffer P2; 10 min) was then added to the re-suspended bacterial cells and mixed

thoroughly by vigorous inversion (5 times). Following a 5-minute incubation at room temperature, lysates were neutralised by the addition of ice-cold Buffer P3 (10 ml), incubated on ice for 20 min, and centrifuged for 30 min (6,000*g*, 4°C) to remove precipitated material (genomic DNA, proteins, cell debris). Supernatant was then transferred to polypropylene tubes and re-centrifuged (3,400*g*, 15 min, 4°C) to remove any residual precipitated material. Supernatant was then applied to a QIAGEN-tip™ 500 column that was previously equilibrated by washing it with Buffer QBT (10 ml). Plasmid DNA was allowed to bind to the column, which was thoroughly washed by applying 60 ml of wash buffer (Buffer QC). Plasmid DNA was then eluted from the column by the addition of 15 ml of Buffer QF (elution buffer) and then 10.5 ml of isopropanol immediately added to the eluate and mixed thoroughly to precipitate DNA. Isopropanol-precipitated DNA was centrifuged (4,000*g*, 30 min, 4°C) and pelleted DNA was washed with 5 ml of room-temperature ethanol (70% v/v). Following centrifugation for 10 min (4,000*g*, 4°C), supernatant was carefully decanted and DNA pellet was air-dried before gently re-dissolving it with nuclease- and DNA-free sterile water to a final concentration of 1-2 mg ml^{-1}. Plasmid quantity and quality was assessed by UV spectrophotometry and agarose gel electrophoresis, as described in *Section 11.16.1*.

11.16.3. Purification of PCR Products

PCR products were purified using the QIAquick™ PCR Purification kit (Qiagen), according to the manufacturer's instructions. Following PCR amplification, 250

µl of Buffer PB were added to the PCR sample (5 volumes buffer for 1 volume of PCR sample) and mixed thoroughly prior to application to a QIAquick™ column. Following centrifugation for 1 min at 14,000*g*, the column was then washed with 0.75 ml of Buffer PE and centrifuged for 1 min at 14,000*g*, followed by an additional centrifugation step (1 min at 14,000*g*) to remove residual wash buffer. DNA was then eluted from the column by adding 30 µl 10 mM Tris, pH 8.5 at room temperature for 2 min and centrifugation at 14,000*g* for 1 min to collect the eluted PCR products. DNA concentration of the samples was determined by UV spectrophotometric analysis over a 230-300 nm absorbance range and absorbance 260 nm/280 nm ($A_{260/280}$) ratio values between 1.8 and 2.0 were considered to be optimal and indicative of pure DNA preparations. DNA samples were stored at -20°C for subsequent analysis.

11.16.4. Genomic DNA Extraction

Peripheral venous blood was obtained from consenting donors, collected into disodium EDTA (K_2EDTA)-containing tubes (S-Monovette™, Sarstedt, Leicester, Leicestershire, UK) and stored at -20°C until processed for genomic DNA extraction. DNA was extracted using QIAamp™ Blood Midi Kit (Qiagen), based on manufacturer's recommendations. In detail, 2 ml of anticoagulated blood was mixed with QIAGEN Protease suspension (200 µl), before the addition of lysis buffer (Buffer AL; 2.4 ml). Adequate cell lysis was ensured by vigorous agitation and vortexing of the mixture for at least 1 min. Lysed blood was incubated at 70°C for 10 min and 2 ml of molecular biology-grade ethanol (100% v/v) was added, followed by vigorous shaking and vortexing of the

samples to achieve even mixing and then applied onto the QIAamp™ Midi column and centrifuged for 3 min at 1,850*g*. The column was then washed using 2 ml of Buffer AW1 and centrifuged for 1 min at 3,700*g*. A second washing step included the addition of 2 ml of Buffer AW2, followed by centrifugation for 15 min at 3,700*g*, to ensure complete removal of the washing buffer. Genomic DNA was eluted from the column by adding Buffer AE (300 µl) to the centre of the column. Then, column was incubated for 5 min at room temperature prior to centrifugation for 2 min at 3,700*g*. Eluted material was then re-applied to the column and eluted as described above to increase the final concentration of the eluted DNA. Extracted genomic DNA concentration of the samples was then determined by spectrophotometric analysis over a 230-300 nm absorbance range using a NanoDrop™ 1000 UV spectrophotometer (Thermo Scientific) and absorbance 260 nm/280 nm ($A_{260/280}$) ratio values between 1.8 and 2.0 were considered to be optimal and indicative of pure DNA preparations, without evidence of protein contamination. DNA samples were stored at -20°C for subsequent analysis.

11.17. Bacterial Transformation

E.coli JM109 or DH5α were transformed using a modification of the previously described calcium chloride protocol (Maniatis *et al.*, 1982). Under aseptic conditions, a single *E.coli* colony from a freshly streaked LB plate was inoculated to pre-warmed (37°C) LB medium (5 ml) and incubated overnight at 37°C shaking (250 rpm). Overnight cultures were then diluted at 1:100 with

fresh, pre-warmed LB medium (200 µl of overnight culture to 19.8 ml LB medium) and cultures were incubated at 37°C. Cell growth was monitored at 40 min intervals by recording the OD_{595} absorbance and incubated until cells reached a mid-exponential growth (OD_{595}=0.7). Then, 1 ml of culture was transferred to individual 1.5 ml microcentrifuge tubes and cells were pelleted by brief centrifugation (14,000*g*, 1 min). Bacterial cells were re-suspended in ice-cold, sterile 50 mM $CaCl_2$ solution (0.5 ml) and incubated on ice for 10 min. Cells were centrifuged and pellets were again re-suspended in ice-cold $CaCl_2$ solution (50 mM; 0.3 ml). Following a 30-minute incubation on ice, plasmid DNA, or sterile water, or ligation reaction products or site-directed mutagenesis products were added to the cells and mixed very gently. Cells were incubated for a further 30 min on ice and heat-shocked by a 2-min incubation in a 42°C water bath. Cells were immediately placed on ice and incubated for 10 min. LB medium (without any selection antibiotics) was then added (1 ml) and cells were incubated for 60 min at 37°C. Then, cells were centrifuged, re-suspended in LB medium (100 µl) and inoculated onto pre-warmed, selective LB plates (50 µl of cell suspension per plate). Plates were incubated in a humidified incubator (37°C) for at least 20 h and any selected bacterial colonies were purified by culturing them into fresh selective plates prior to further plasmid analysis.

11.18. Short Hairpin RNA (shRNA)-Mediated CD32 Expression Knock-Down

CD32a expression was downregulated using shRNA lentiviral vectors that were obtained from Sigma Aldrich (MISSION shRNA clones; for more information, see *Section 11.3*). All five shRNA plasmid clones supplied were validated for CD32 knockdown efficiency by transient transfection to CHO cells expressing wild type CD32a. One particular clone achieved >70% gene expression knock-down (clone TRCN029578; recognising sequence 5'-GAAGAAACCAACAATGACTAT) as evidenced by flow cytometric analysis of surface levels of CD32a expression. This clone was then stably transfected to wild type K562 cells as described in *Section 11.21*. Following selection and cell sorting, CD32a expression and IgG complex binding was assessed in shRNA expressing K562 cells.

11.19. Site-Directed Mutagenesis

11.19.1. Design of Mutagenic Primer Pairs

For the site-directed mutagenesis, primers were designed to incorporate the relevant mutations based on the following criteria: (i) GC content of at least 55% with either C or G at both 5' and 3' primer ends, (ii) ≥90% primer purity; primers were purified by HPLC (high performance liquid chromatography) (MWG Biotech), (iii) mismatched sequences were designed to be at the centre of the primer with at least 10 bp sequence flanking the mutation at each end

and (iv) primer T_m of at least 70°C. For each mutation, a set of two primers was designed, each covering the sense and the anti-sense strand of the plasmid at the same sequence.

The following primer sequences were designed and used in this study: For the A224S mutation: Forward: 5'-GGCTGTGGTCATTTCGACTGCTGTAGC-3', Reverse: 5'-GCTACAGCAGTCGAAATGACCACAGCC-3'. For the C241A mutation: Forward: 5'-GTAGTGGCCTTGATCTACGCCAGGAAAAAGCGGATTTC-3', Reverse: 5'-GAAATCCGCTTTTTCCTGGCGTAGATCAAGGCCACTAC-3'. For the introduction of the *Xho*I restriction site (see *Section 11.20* for more details): Forward: 5'-CCAAGTGCCCAGCATGGGCTCGAGTTCACCAATGGGGATC-3', Reverse: 5'-GATCCCCATTGG TGAACTCGAGCCCATGCTGGGCACTTGG-3'. For the generation of silent mutations in the shRNA target sequence (see *Section 11.18* for more details): Forward: 5'-GAAGAAACCAACAACGATTACGAAACAGCTGACGGCGG-3', Reverse: 5'-CCGCCGTCAGCTGTTTCGTAATCGTTGTTGGTTTCTTC-3'. All primers were synthesised and obtained from MWG Biotech/Eurofins.

11.19.2. Site-Directed Mutagenesis

Full length human CD32a ORF sub-cloned into a pSELECT-neo-mcs vector was obtained from Invivogen and was used as the template for the site-mutagenesis reactions. Specific primers incorporating the desired mutations were designed and obtained from MWG Biotech - Eurofins (RP-HPLC purified, >90% purity). For more information, see *Section 11.19.1.* Each site-directed mutagenesis reaction (50 µl) contained 100 ng plasmid DNA as template, 200

μM dNTP (Promega), 125 ng of each primer pair and 2.5 U of *Pfu*Ultra™ High Fidelity DNA polymerase in the presence of the respective reaction buffer (Stratagene). Reactions were performed in a Touchgene™ gradient thermal cycler (Techne Corporation, Fisher Scientific UK, Loughborough, Leicestershire, UK) and cycling conditions were the following: 1 min denaturation at 94°C, 18 cycles of 1 min at 94°C, 1 min at 55°C, 9 min at 68°C, followed by an additional 30-minute extension step at 68°C. Each reaction was incubated for 3 h at 37°C with *Dpn*I (0.2 U μl^{-1}; Promega) to digest the methylated wild-type plasmid and mutated plasmids were then transformed into $CaCl_2$-competent *E.coli* DH5α cells (see *Section 11.17* for bacterial transformation). Mutated plasmid sequences were validated by direct sequencing and, where applicable by restriction endonuclease analysis.

11.19.3. Plasmid DNA sequencing

The efficiency and validity of the site-directed mutagenesis reactions was validated by the direct sequencing of the mutated plasmid sequences. DNA sequencing reactions were performed by the Sequencing Service (College of Life Sciences, University of Dundee, UK) using Applied Biosystems Big-Dye™ 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer (Applied Biosystems, Birchwood, Warrington, Cheshire, UK). For the sequencing, the following set of primer pairs were designed and used, so as to cover the entire plasmid sequence (4.5 kb): For the sense strand: Forward 1: 5'- CCTGCTTGCTCAACTCTACG-3', Forward 2 5'-GAGCGACTCCATTCAGTGGT-3', Forward 3 5'-CAGCCAATTCCACTGATCCT-3', For

the anti-sense strand: Reverse 1 5'-CTGTCAATGGTTGAAGCAGC-3', Reverse 2 5'-ATGTGACCTTGACCAGAGGC-3', Reverse 3 5'- ACTGTTGACATGGTCGTTGG-3'. Plasmid DNA was sequenced at both the sense and the antisense strands (double-pass sequencing) and chromatograph files were viewed and analysed using the Sequence Scanner Software (v1.0) (Applied Biosystems) or the 4Peaks™ Sequence Analysis Software (Mekentosj.com, Aalsmeer, The Netherlands).

11.20. Generation of GPI-anchored CD32

11.20.1. Design of the GPI-anchored CD32

The GPI modification site was predicted in the human CD55 gene based on Eisenhaber *et al.* (1999) and using the big-PI Predictor Software (http://mendel.imp.ac.at/gpi/gpi_server.html). The chimaeric CD32/55 construct was designed to include the following: (i) the extracellular, IgG binding domains of CD32, consisting of the signal peptide, the two immunoglobulin domains and the extracellular, membrane proximal region and (ii) the GPI-anchored consensus sequence of CD55, comprising the ω site and downstream consensus domains. Hydrophobicity and protein folding thermodynamic analyses were also performed on the designed protein sequences using the PreLink software (http://genomics.eu.org/prelink) based on Coeytaux and Poupon (2005), in order to minimise the possibility of protein misfolding of the engineered CD32/55 construct.

11.20.2. Cloning of the CD32/55 Chimaeric Protein

The GPI-anchored CD32 (CD32/55 chimaeric protein) was designed as described in *Section 11.20.1*. CD55 GPI-consensus sequence was sub-cloned to a pJ201 vector and the *Xho*I (Restriction site sequence) and *Bln*I (*Avr*II) (restriction site) sites were engineered to flank the 5' and 3' end, respectively. The design and selection of the chosen restriction sites was based on the following criteria: (i) creation of cohesive ends (either in the form of 5' or 3' overhangs), (ii) absence of these restriction recognition sites in the sequence of the pJ201 vector and of the pSELECT-neo-mcs-*FCGR2A* plasmid, unless in designated or intentionally created sites, (iii) introduction of these restriction sites in the pSELECT-neo-mcs-*FCGR2A* plasmid by site-directed mutagenesis using the minimum number of nucleotide changes. The final construct was synthetically generated from DNA2.0.

An *Xho*I site was introduced at the end of the extracellular domain of the wild-type CD32a ORF (pSELECT-neo-mcs-*FCGR2A* plasmid) by site-directed mutagenesis (see *Sections 11.19.1 and 11.19.2* for primer design and site-directed mutagenesis protocol). The *Bln*I (*Avr*II) restriction site was present in the original pSELECT-neo-mcs-*FCGR2A* plasmid, within the CD32a ORF and downstream of the introduced *Xho*I recognition site. Cloning of the CD55 GPI-consensus sequence within the CD32 ORF (pSELECT-neo-mcs-*FCGR2A* plasmid comprising the *Xho*I site) was performed using the Rapid DNA dephos™ and ligation kit (Roche Applied Science), following manufacturer's recommendations.

Initially, the purified pJ201:CD55-GPI plasmid was restricted with *Xho*I (Promega) and *Bln*I (Roche Applied Science) for 3 h at 37°C, according to product instructions, so as to excise the CD55 GPI-consensus insert from the pJ201 vector. Next, the restriction products were resolved by gel electrophoresis, using ultra pure, low melting point agarose (1% w/v) without the addition of ethidium bromide. Following electrophoresis, gels were briefly incubated with ethidium bromide solution (0.2 μg ml^{-1}; 5 min), following by wash in ultra pure water for 20 min. Gels were visualised under low-power UV light and the CD55-GPI insert was excised and purified from the gel, using the QIAquick™ Gel extraction kit (Qiagen), based on manufacturer's instructions. Briefly, excised gel fragments were dissolved by the addition of Buffer QC (3 volumes for 1 volume gel), following by incubation at 50°C for 10 min or until all agarose was dissolved. Then, isopropanol was added at equal volume to the gel and samples were vigorously agitated to ensure even mixing. Samples were then applied to a QIAquick™ column and centrifuged for 1 min (14,000*g*). The flow-through fraction was discarded and column was washed with Buffer QG (0.5 ml) and Buffer PE (0.75 ml). At each washing step, the column flow-through was discarded from the collection tube and column was centrifuged for an additional 1 min at 14,000*g* to completely remove residual wash buffer. DNA was then eluted by incubation for 1 min at room temperature with 30 μl 10 mM Tris, pH 8.5 and centrifugation at 14,000*g* for 1 min. The purified DNA fragment was quantified by UV spectrophotometry and stored at -20°C, until used for the ligation reaction.

The pSELECT-neo-mcs-*FCGR2A* plasmid vector containing the *Xho*I restriction site was restricted with *Xho*I (Promega) and *Bln*I (Roche Applied Science) for 3 h at 37°C. A small fraction of the restriction products (150 ng) was analysed by agarose gel (1% w/v) electrophoresis to assess the efficiency and specificity of the reaction and the remaining of the restricted pSELECT-neo-mcs-*FCGR2A* plasmid vector was purified using the QIAquick™ PCR Purification Kit (Qiagen), so as to remove restriction enzymes that will interfere with the ligation reaction. The detailed protocol of the DNA purification using the QIAquick™ PCR Purification Kit (Qiagen) is described in *Section 11.16.3*. The purified and restricted pSELECT-neo-mcs-*FCGR2A* vector (500 ng) was then treated with alkaline phosphatase (1 U) for 45 min at 37°C using the Rapid DNA Dephos™ Kit (Roche Applied Science). Alkaline phosphatase was heat-inactivated (2 min; 75°C) and 50 ng of vector DNA from the dephosphorylation reaction mixture was used for the ligation reaction. Ligation was performed using the Roche DNA Ligation Kit (Roche Applied Science) and reaction mixtures were assembled as instructed by the manufacturer. Insert DNA (excised and purified CD55-GPI consensus fragment) was added to the ligation reaction at a final molar ratio of insert:vector of 3:1.

Ligation mixtures were incubated at room temperature for 90 min and ligation products (5 µl) were transformed into $CaCl_2$-competent DH5α cells and plated onto selective LB medium. Any positive (resistant) clones were purified, plasmids were extracted and their inserts were analysed by double *Xho*I-*Bln*I restriction. Plasmids with the predicted insert size were selected and their

sequences were validated by direct sequencing, as described in *Section 11.19.3*. CD32/55 expressing plasmid was then transfected in CHO-K1 and K562 cells (described in detail in *Section 11.21*) and the GPI modification of the chimaeric protein was confirmed by reduction in expression following treatment with phosphatidylinositol-specific phospholipase C (PI-PLC; 0.1 U ml^{-1}) (Sigma Aldrich) for 20 min at 37°C.

11.21. Generation of Transgene-Expressing Cell Lines

11.21.1. Cell Transfection

CHO cells were transfected using either jetPEI™ Transfection reagent (PolyPlus Transfection) or Lipofectamine LTX™ (Invitrogen), according to manufacturer's instructions. For jetPEI™-based transfection, 24 h before transfection, $3x10^5$ cells were plated in each well of a 6 well plate and cultured in DMEM:F-12 (1:1) supplemented with GlutaMAX™ and 10% (v/v) FBS. Purified plasmid DNA (3 µg) was diluted in 150 mM NaCl to a final volume of 100 µl. Similarly, 3.6 µl of jetPEI™ transfection reagent was diluted in NaCl (150 mM) to a final volume of 100 µl and following gentle vortexing it was added to the diluted DNA solution. The mixture was immediately vortexed and incubated at room temperature for 30 min. Then, 200 µl of the jetPEI™/DNA mixture was added drop-wise to the cells, which contained 2 ml of serum containing medium (DMEM:F-12 (1:1) supplemented with GlutaMAX™ and 10% (v/v) FBS) and the plate was gently shaken to ensure adequate mixing.

Cells were incubated at 37°C (5% CO_2) and expression was assessed at least 24 h following transfection.

For Lipofectamine-based transfection, the following protocol was used: CHO cells were plated at $2x10^5$ cells per well in a 6-well plate 24 hours before transfection and cultured in DMEM:F-12 (1:1) supplemented with GlutaMAX™ and 10% (v/v) FCS. Plasmid DNA (2.5 µg) was diluted in 500 µl Opti-MEM® I Reduced growth medium (Invitrogen) and 8.75 µl of Lipofectamine LTX™ reagent was added. Lipofectamine-DNA solution was gently mixed and incubated at room temperature for 25 min. In the meantime, culture medium from cells was replaced with 2 ml of complete growth medium, containing 10% (v/v) FCS, but without any antibiotics. DNA-Lipofectamine complexes (500 µl) were then added drop-wise to each well and mixed gently. Cells were then incubated at 37°C (5% CO_2) and expression was assessed 24 h following transfection by flow cytometry.

K562 cells were transfected using Lipofectamine LTX™ and Lipofectamine PLUS™ reagent (Invitrogen) based on the following protocol: $5x10^5$ cells were plated into each well of a 6 well tissue culture plate containing 2 ml of RPMI 1640 complete medium (2 mM L-glutamine + 10% (v/v) FCS). Plasmid DNA (2.5 µg) was diluted in 500 µl Opti-MEM® I Reduced growth medium (Invitrogen) and 2.5 µl of PLUS™ reagent were added to the diluted DNA, followed by gentle mixing and incubation for 15 min at room temperature. Then, 10 µl of Lipofectamine LTX was added to the DNA/PLUS™ solution, mixed gently and incubated for 25 min at room temperature to allow

formation of Lipofectamine/DNA complexes. This mixture (500 µl) was then added drop-wise to the plated cells, mixed gently and cells were incubated at 37°C (5% CO_2). Transgene expression was assessed 24 h following transfection by flow cytometry as described in *Section 11.6*.

11.21.2. Cell Selection

For the generation of stable transfectants, 48 h following transfection cells were cultured with the corresponding selection antibiotic. CHO cells were cultured in DMEM:F12 (1:1) (GlutaMAX™ + 10% (v/v) FCS) medium containing 1 mg ml^{-1} G418 (Invitrogen) for about 14 days. K562 cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% (v/v) FCS and selected with either G418 (initially added at 1 mg ml^{-1} for the first 48 h and then at 500 µg ml^{-1}) or puromycin (2 µg ml^{-1}; Sigma Aldrich). Selection of positive clones was performed by cell sorting (see *Section 11.21.3* for more details). For CHO cells, single clone populations were established either by serial dilution culture or single cell sorting so as to maintain a stable transgene-expressing cell population.

11.21.3. Cell Sorting

Following direct immunolabelling of cells with FITC-conjugated anti-CD32 antibodies (Clone FLI8.26, $IgG2_b$, BD Biosciences)(for detailed protocol on immunolabelling see *Section 11.6*), cells were re-suspended in DMEM medium supplemented with 2 mM L-glutamine and 0.5% (v/v) FCS and processed

immediately for fluorescence-activated cell sorting using a BD FACS Vantage™ SE/DiVA™ Cell sorter. Sorted cells were collected in either 5 ml polypropylene tubes (BD Biosciences) for K562 cells or in 96-well plates (Costar) for CHO cells, using single cell sorting so as to obtain single clone cell populations. Data were visualised and analysed using BD FACS Diva™ software (BD Biosciences).

11.22. Assessment of CD32-Lipid Raft Interactions

11.22.1. Detergent-Resistant Membrane Fractionation and Analysis

Detergent-resistant membrane (DRM) domain fractionation was performed essentially as previously described based on well-validated protocols (Rollet-Labelle *et al.*, 2004; Setiadi and McEver, 2008). All cell lysis procedures were carried out at 4°C and unless otherwise stated, all reagents were ice cold. Following stimulation of cells (CHO: $2x10^7$ or K562: $1x10^7$) with IgG complexes (hHAIgG, 10 μg ml^{-1}), cells were washed twice with ice-cold PBS (Ca^{2+}/Mg^{2+} free) and incubated for 10 min with TNE buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA; pH 7.5) at 4°C. Cells were homogenised using a 25-gauge needle (passed 15-20 times) and incubated on ice for a further 5 min prior to the addition of Triton™ X-100 (0.25% v/v final concentration).

Triton™ X-100 lysates were incubated for 30 min on ice before addition of OptiPrep™ (Axis-Shield; Sigma Aldrich) density gradient medium to a final concentration of 40% (v/v). After gentle mixing, lysates (600 μl) were applied to the bottom of pre-chilled 2.2 ml ultracentrifuge, thin-walled, polypropylene

tubes (Ultra-Clear™, 8x49 mm, Beckman Coulter, High Wycombe, Buckinghamshire, UK) and sequentially overlaid with 35% (v/v) and 25% (v/v) OptiPrep™ solutions (600 µl in TNE buffer), followed by 200 µl of TNE buffer (0%). Samples were then centrifuged at 54,000 rpm (194,000g_{avg}) for 2 h at 4°C in an Optima-MAX™ benchtop ultracentrifuge (Beckman Coulter) using a TLS-55 swinging bucket rotor (Beckman Coulter). Following centrifugation, fractions (200 µl) were carefully collected from the top to the bottom of the tube and stored at -20°C for subsequent analysis.

Fractions obtained after DRM isolation were resolved by SDS-PAGE using 4-12% Bis-Tris gradient gels (NuPAGE, Invitrogen) under non-reducing conditions, according to manufacturer's instructions. Following the same recommendations, proteins were then electroblotted onto nitrocellulose membranes (Amersham Biosciences)(30 V, 60 min) and blocked with 5% w/v fat-free milk in PBS containing 0.05% (v/v) Tween™-20. Membranes were probed for 60 min at room temperature with the corresponding primary antibodies: goat anti-human CD32a (1:500, R&D Systems), mouse anti-human caveolin-1 (1:500, BD Biosciences), mouse anti-human transferrin receptor (CD71)(1:1000, BD Biosciences), mouse anti-human flotillin-1 (1:1000, BD Biosciences). Membranes were then incubated with HRP-conjugated secondary antibodies (either goat anti-mouse or rabbit anti-goat Ig; 1:5000, Dako Cytomation) for 40 min at room temperatures. All antibody dilutions were prepared in PBS containing Tween™-20 (0.05% v/v) and after each incubation step with either primary or secondary antibody, membranes were

thoroughly washed at least four times with PBS-Tween™-20 (0.05% v/v). Bound antibodies were visualised using enhanced chemiluminescence (ECL™ or ECL Plus™ reagent; Amersham Biosciences) and Hyperfilm™ (Amersham Biosciences; GE Healthcare). For the quantification of gel band intensities, ImageJ (National Institutes of Health, Bethesda, Maryland, USA) software package was used.

11.22.2. Confocal Immunofluorescence Microscopy

CD32 and GM1 immunolabelling was performed using previously described protocols (Harder *et al.*, 1998). Briefly, CHO cells were grown on sterile glass coverslips for 24 h (37°C; 5% CO_2) prior to immunolabelling. Cells were washed twice with ice-cold medium (DMEM containing 2 mg ml^{-1} BSA) and incubated with the mouse anti-human CD32 antibody (IV.3 clone; 20 μg ml^{-1}) and biotin-conjugated cholera toxin B subunit (10 μg ml^{-1}) at 12°C for 60 min under gentle rocking. Then, cells were washed with ice-cold PBS (Ca^{2+}/Mg^{2+} free) and subsequently incubated with Alexa Fluor™ 647-labelled streptavidin (10 μg ml^{-1}) and R-PE-conjugated goat $F(ab')_2$ anti-mouse IgG (10 μg ml^{-1}). Cells were fixed on ice for 4 min with 3.8% (w/v) formaldehyde in PBS (Ca^{2+}/ Mg^{2+} free) followed by a 5-minute incubation in methanol (100%) at -20°C. K562 immunolabelling was performed as described above with the exception that cells were labelled in suspension and were then attached to poly-L-lysine (Sigma Aldrich)-coated coverslips, according to manufacturer's instructions prior to fixation. Coverslips were briefly washed in distilled water and then mounted using the FluorSave™ reagent (Merck-Calbiochem). Slides were

visualised (100x oil-immersion objective) using Zeiss™ LSM510meta laser scanning confocal microscope (Carl Zeiss, Welwyn Garden City, Hertfordshire, UK) equipped with argon and helium/neon lasers and digital images were prepared using the LSM™ image browser (Carl Zeiss), Volocity™ (Improvision – PerkinElmer, Waltham, Massachusetts, USA) and Adobe® Photoshop® CS4 (Adobe Corporation, San Jose, California, USA). For the quantification of CD32-GM1 co-localisation, images (from at least 50 cells obtained from random fields) were analysed using the Zeiss® LSM™ 510 software package or Volocity™ and co-localisation expressed as the percentage of pixels from the CD32 channel co-localising with pixels from the GM1 channel.

11.23. Measurement of Cellular Cholesterol

Total cellular cholesterol was quantified in CHO and K562 cells, using the cholesterol/cholesteryl ester quantitation kit (Merck Calbiochem), according to manufacturer's instructions. Cells ($5x10^6$) were washed twice with ice-cold PBS (Ca^{2+}/Mg^{2+}-free) and pellets were air-dried to ensure complete removal of PBS. Then, cell pellets were re-suspended in 1ml chloroform:methanol (2:1) solution and homogenised using a 25-gauge needle and by sonication, until completely dissolved. Samples were then centrifuged for 10 min at 14,000*g* and the organic phase was transferred into a glass tube and vacuum-dried using a heated vacuum centrifuge (Eppendorf, Cambridge, Cambridgeshire, UK). Dried lipids were re-dissolved in 100 µl isopropanol containing 10% (v/v) Triton™ X-100 and stored at -20°C until further analysis. Samples (1 µl)

were then diluted with the supplied Cholesterol Reaction Buffer (49 µl) and co-incubated for 60 min at 37°C with an equal volume of the reaction mix, containing the fluorescent cholesterol probe, cholesterol esterase and cholesterol oxidase. During this 60-minute incubation step, cholesteryl esters are hydrolysed by cholesterol esterases to yield cholesterol, which is then oxidised by cholesterol oxidase to produce hydrogen peroxide (H_2O_2). Hydrogen peroxide then reacts with the sensitive cholesterol probe resorufin, which becomes fluorescent. Sample fluorescence was quantified at an excitation wavelength of 535 nm and an emission wavelength of 590 nm, using a BioTek™ microplate fluorimeter – spectrophotometer (BioTek Instruments). Standard curves were generated using predefined amounts of purified cholesterol (0-1 µg) and results are defined as the mean cholesterol amount of two independent experiments performed in triplicates.

11.24. SNP Genotyping

11.24.1. Allele-specific PCR Amplification

For the determination of FcγRIIIb NA1/2 and FcγRIIa R131H polymorphisms, PCR amplification reactions were performed using allele-specific PCR primers (Eurofins, MWG). Nucleotides in bold are allele-specific and those underlined indicate mismatched nucleotides to the original gene sequences to increase primer specificity. For the *FCGR3B* NA1 allele: forward: 5'-CAGTGGTTTCACAATGTGA**A**-3', reverse: 5'-ATGGACTTCTAGCTGCACCG-3'; For the *FCGR3B* NA2 allele: forward: 5'-CTCAATGGTACAG**C**GTGCTT-3', reverse: 5'-

CTCAATGGTACAGCGTGCTT-3'. For the *FCGR2A* R131 allele: forward: 5'-AAATCCCAGAAATTCTCACG-3', reverse: 5'-CACTCCTCTTTGCTCCAGTG-3'; For the *FCGR2A* H131 allele: forward: 5'-AAATCCCAGAAATTCTCACA-3', reverse: 5'-CACTCCTCTTTGCTCCAGTG-3'.

PCR amplification reactions were performed in a 50-µl volume, containing 200 µM dNTP, 1.5 mM $MgCl_2$, 200 nM of each primer pair (forward and reverse), 2.5 U *Taq* DNA polymerase (Go*Taq*™ Flexi DNA Polymerase, Promega) in green Go*Taq*™ Flexi buffer (Promega) and 1 µl of extracted genomic DNA (200-500 ng). Reactions were performed in a Touchgene™ gradient thermal cycler (Techne). All PCR amplification conditions included an initial denaturation step of 5 min at 94°C, and a final extension step of 5 min at 72°C and were as following: for *FCGR2A* R131 and H131: 30 cycles of 45 sec at 94°C, 45 sec at 54°C and 45 sec at 72°C; for *FCGR3B* NA1: 11 cycles of 1 min at 94°C, 1.5 min at 60°C and 2.5 min at 72°C, followed by 25 cycles of 1 min at 95°C, 1 min at 57°C and 1 min at 72°C; for *FCGR3B* NA2: 36 cycles of 1 min at 95°C, 1 min 62°C and 1 min at 72°C.

PCR products (10 µl) were analysed by agarose gel (2% w/v; UltraPure™ Agarose, Invitrogen) electrophoresis in Tris Borate EDTA (TBE) buffer. Gels were stained with ethidium bromide (added to the gel at a final concentration of 1 µg ml^{-1}) and visualised under UV illumination. The efficiency and specificity of the allele-specific PCR amplification was further validated by direct sequencing (see *Section 11.24.2* for more details).

11.24.2. DNA Sequencing of PCR Products

In order to validate the efficiency and specificity of the allele-specific PCR amplification, regions within the *FCGR2A* and *FCGR3B* genes containing the R131H and NA1/NA2 polymorphisms, respectively were PCR amplified in a 50 µl reaction volume containing 200 µM dNTP, 1.5 mM $MgCl_2$, 200 nM of each primer pair (forward and reverse), 2.5 U *Taq* DNA polymerase (Go*Taq*™ Flexi DNA Polymerase, Promega) in colourless Go*Taq*™ Flexi buffer (Promega) and 1 µl of extracted genomic DNA (200-500 ng). The following primer pairs were used: for FcγRIIa: Forward: 5'-TGAGACTGAAAAACCCTTGG-3', Reverse: 5'-CACTCCTCTTTGCTCCAGTG-3'; For FcγRIIIb: Forward: 5'-GTGTAGAGCCTGCTCCTCTCC-3', Reverse: 5'-AGTGGGACCACACATCATCTC-3'.

Reactions were performed in a Touchgene™ gradient thermal cycler (Techne). All PCR amplification conditions included an initial denaturation step of 5 min at 94°C, and a final extension step of 5 min at 72°C and were as following: for *FCGR2A*: 35 cycles of 45 sec at 94°C, 45 sec at 54°C and 45 sec at 72°C; for *FCGR3B*: 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. In order to confirm successful amplification, an aliquot (10 µl) of the PCR products was analysed by agarose gel (2% w/v; UltraPure™ Agarose, Invitrogen) electrophoresis and the remaining PCR samples were purified using the QIAquick™ PCR Purification kit (Qiagen)(see *Section 11.16.3* for further details) prior to direct sequencing analysis.

DNA sequencing reactions were performed by the Sequencing Service (College of Life Sciences, University of Dundee, UK) using Applied Biosystems Big-Dye™ 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. For the sequencing, the same set of primer pairs were used with those used for the PCR amplification (see above) and both the sense and the antisense strands of the PCR product were sequenced (double-pass sequencing). Chromatograph files were viewed and analysed using the Sequence Scanner Software (v1.0) (Applied Biosystems) or the 4Peaks™ Sequence Analysis Software (Mekentosj).

11.24.3. Allotype-specific Neutrophil Immunolabelling and Flow Cytometry

Neutrophil granulocytes were isolated by dextran sedimentation and discontinuous Percoll™ gradient centrifugation (described in *Section 11.5.2*; (Bournazos *et al.*, 2008; Dransfield *et al.*, 1995)) from citrated peripheral venous blood drawn from subjects previously typed as NA1/NA1, NA1/NA2, and NA2/NA2. Eosinophils comprised ≤3% of the isolated polymorphonuclear cell fraction. Neutrophils were immunolabelled using either allotype-specific mouse monoclonal antibodies (10 μg ml^{-1}) against human FcγRIIIb (NA1: CLB-gran/11, mouse $IgG2_a$, NA2: GRM1, mouse $IgG2_a$) or isotype negative control monoclonal antibody (UPC-10, mouse $IgG2_a$; Sigma Aldrich). Briefly, freshly isolated cells were incubated with the corresponding monoclonal antibody (10 μg ml^{-1} diluted in PBS containing 0.1% w/v BSA) or the isotype control antibody for 30 min on ice at a final concentration of 10^5 cells ml^{-1}. Cells were then washed twice with PBS containing 0.1% (w/v) BSA and incubated with

Alexa Fluor™ 488-conjugated F(ab')$_2$ goat anti-mouse Ig (Invitrogen) for 20 min on ice. Cells were again washed twice with PBS containing 0.1% (w/v) BSA and re-suspended in the same buffer at a final concentration of $2x10^5$ cells ml^{-1}, prior to flow cytometric analysis. Surface expression of FcγRIIIb (NA1 or NA2) was assessed by flow cytometry based on FL-1 intensity fluorescence using a BD FACScan™ flow cytometer (BD Biosciences) and data were analysed using BD CellQuest™ (BD Biosciences), BD FACS Diva™ (BD Biosciences) or FlowJo™ (TreeStar) software.

11.25. Measurement of *FCGR3B* Copy Number Variation

11.25.1. Quantification of FCGR3B Copy Number by Quantitative Real-Time PCR (qPCR)

FCGR3B gene copy number was measured by quantitative real-time PCR, based on previously described protocols (Aitman *et al.*, 2006; Fanciulli *et al.*, 2007; Willcocks *et al.*, 2008). PCR amplification reactions were performed using QuantiFast® SYBR Green PCR Kit (Qiagen) in 25 µl volume, containing 12.5 µl of 2x QuantiFast® SYBR Green PCR Master Mix (Qiagen), 1 µM of each primer pair, and 2.5 ng of genomic DNA. The following primer pairs were used: for *FCGR3B*: Forward: 5'-CACCTTGAATCTCATCCCCAGGGTCTTG-3'; Reverse: 5'-CCATCTCTGTCACCTGCCAG-3'; For *CD36* (used as a single copy control): Forward: 5'-TAAGTTCAGGTTCCTGGAATGC-3'; Reverse: 5'-CAAATTATGGTATGGACTGTGC-3'.

Reactions were performed in 96-well PCR plates (MicroAmp® Fast 96-well Reaction Plates, Applied Biosystems) using an Applied Biosystems 7500 Fast (Applied Biosystems) real-time cycler. Amplification conditions were the following: initial denaturation and polymerase activation step for 5 min at 95°C followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C. Melting curve analysis of the PCR products was performed to verify their specificity and identity. Standard curves were generated by serial two-fold dilution of a single genomic DNA sample over the range of 25 ng to 0.78 ng per reaction. Unless otherwise stated, samples and standard curve reactions were run in quadruplicates. Amplification data were collected and analysed using the Sequence Detection System (SDS®) software (v1.4)(Applied Biosystems).

For the analysis of results, baseline amplification threshold and threshold cycle (Ct) values were automatically calculated by the SDS® software. Samples that displayed deviations from the expected melting curve analysis profile, suggesting non-specific amplification, were excluded from analysis. Based on the standard curve analysis, Ct values from each reaction were expressed as amount of DNA (ng). The reproducibility of the qPCR assay for each sample was assessed by calculating the coefficient of intra-sample variation (Coefficient of Variation (CV)%= 100x(standard deviation/mean)) and samples with >15% CV were treated as outliers and excluded from the dataset. The mean amount of DNA for each sample was calculated from quadruplicate reactions and the ratio of *FCGR3B*- to *CD36*-specific amplification was used to determine *FCGR3B* gene copy number for each sample.

11.25.2. Bioinformatics Analysis of Array Comparative Genome Hybridization (aCGH) Data

In order to validate the results obtained from the determination of *FCGR3B* gene copy number variation using qPCR, we compared our results with those obtained from the Whole Genome TilePath (WGTP) project from the Sanger Institute (Redon *et al.*, 2006). This project involved the genome-wide analysis of copy number variation in 270 HapMap individuals using array comparative genome hybridisation (aCGH) on the WGTP array. The aCGH data from the WGTP project were downloaded from the Sanger Institute website (http://www.sanger.ac.uk/humgen/cnv/data/). Log_2 intensity ratio values that were normalised to a single male reference (HapMap individual NA10851) were used for analysis. The probe (8H4), mapping to the Fcγ receptor locus (1*q*23), which also contains the *FCGR3B* gene, has been identified based on the March 2006 human genome assembly (NCBI Build 36.1) using the University of California, Santa Cruz genome browser (http://genome.ucsc.edu). Plotting of the log_2 intensity ratios from the 270 HapMap individuals revealed distinct pattern of clustering, suggestive of groups with 0, 1, 2, 3 and >3 gene copies. Based on these results, two individuals were selected from each group and were the following: for 0 copies: NA18564 and NA19210; for 1 copy: NA18853 and NA10839; for 2 copies: NA12248 and NA19005; for 3 copies: NA12234 and NA10860; and for >3 copies: NA10846 and NA18603. DNA samples were obtained from the Coriell Institute (Camden, New Jersey, USA) and used as described in *Section 11.25.1*, so as to determine *FCGR3B* copy number by qPCR.

11.26. Data Analysis

11.26.1. Statistical Analysis

Results from multiple experiments are presented as mean ± standard deviation (SD). One- or two-way analysis of variance (ANOVA) was used to test for differences in the mean values of quantitative variables, and where statistically significant effects were found, post-*hoc* analysis using Bonferroni *t*-test was performed. Unless otherwise stated, *P* values of <0.05 were considered to be statistically significant. Data were analysed with GraphPad™ Prism software (v5.0b)(GraphPad Software, La Jolla, California, USA).

11.26.2. Pulmonary Function Data Analysis

The following pulmonary function measurements were obtained at baseline: FEV_1, FVC, FEV_1/VC, TLC, D_{LCO} and K_{CO}. Baseline was defined as the date of the first radiologic evidence for IPF (date of first HRCT scan). FVC and D_{LCO} were monitored for at least 12 months following disease diagnosis (baseline) to assess disease progression and prognosis. Predicted pulmonary function values were calculated for each patient based on the gender, age and height, according to the standardised criteria from the European Community for Coal and Steel (ECCS) and the European Respiratory Society (ERS)(*Section 15.3*). Unless otherwise stated, pulmonary function values are expressed as the percentage predicted for each patient. One-way analysis of variance (ANOVA) was used to test for differences in the mean values (actual or % predicted) of pulmonary function measurements among the three genotypes for each SNP

(FcγRIIa R131H or FcγRIIIb NA1/2) or among the FcγRIIIb copy number variants (<2, 2, >2).

Patients with <40% predicted D_{LCO} at baseline are generally associated with a more severe disease phenotype (Bradley *et al.*, 2008; Latsi *et al.*, 2003b). Similarly, a drop of 10% in FVC or 15% in D_{LCO} in the first 12 months following diagnosis is indicative of progressive disease (Bradley *et al.*, 2008; Flaherty *et al.*, 2003; Hanson *et al.*, 1995; Latsi *et al.*, 2003b). Therefore, baseline and serial lung function measurements were used to determine disease severity and progression, and patients were categorised accordingly. Association of genotypes or copy number variants with disease severity (<40% D_{LCO} at presentation) or progression (-10% FVC or -15% D_{LCO}) was assessed by 3x2 contingency tables (χ^2 test with two degrees of freedom (*df*)). Fisher's exact test (2x2 contingency table) was used to assess for differences in the allelic frequency among the disease severity or progression groups. χ^2 test was used to test for association of subject gender with genotypes or disease progression / severity groups. Unless otherwise stated, pulmonary function data are presented as mean ± SD with the exception of graphical representations of lung function measurements, where data are presented as mean ± 95% confidence interval (CI).

11.26.3. Genetic Analysis – SNP

Hardy-Weinberg equilibrium was assessed by a χ^2 test with one degree of freedom (no of alleles – 1) to test for differences between the observed

genotype frequency and the expected one. Expected genotype frequencies were calculated assuming that the genotypes were in Hardy-Weinberg equilibrium, based on the observed allele frequencies. Differences in the genotype frequencies between control and IPF patients were analysed by the χ^2 test with two degrees of freedom (*df*). Fisher's exact test was used to assess differences in allele frequencies between the two cohorts and to calculate the odds ratio that confers protection or susceptibility for a particular allele.

11.26.4. Genetic Analysis – CNV

Association of FcγRIIIb copy number and IPF susceptibility was assessed by two main strategies: (i) direct comparison of *FCGR3B*:*CD36* ratio values between the two cohorts and (ii) assignment of copy number based on *FCGR3B*:*CD36* ratios.

Firstly, non-parametric tests (Mann-Whitney) were used to test for differences in the *FCGR3B*:*CD36* ratio values between control and IPF groups, since data were clustered in distinct copy number groups and were not following Gaussian distribution. Secondly, assignment of the *FCGR3B*:*CD36* ratio to copy number classes was initially attempted by *a priori* binning of ratio values to predefined thresholds (<2: 0-0.75 (*FCGR3B*:*CD36*); 2: 0.75-1.25; >2: >1.25). However, in order to avoid biases in the copy number classification due to the presence of samples in the dataset with *FCGR3B*:*CD36* values near the threshold boundaries, a recently developed, likelihood ratio test was employed, which integrates copy number classification and case-control

association testing (Barnes *et al.*, 2008). The fitting code was obtained from http://cnv-tools.sourceforge.net/ and was available as an R package (http://www.r-project.org/). Clustering and copy number assignment of the *FCGR3B*:*CD36* ratios were performed with two fitting models: principal components analysis (PCA) and linear discriminant function (LDF), as previously described. Posterior probability values for each classification component (P1, P2, P3 representing 1, 2 and 3 copies, respectively) of the samples are presented in *Section 15.2*. There was agreement in the copy number classification between PCA- and LDF-transformed data. Association between copy numbers and the case-control status was tested using the likelihood ratio approach, using the Gaussian or T mixture model and the results were distributed as χ^2 with one degree of freedom, as described by Barnes and co-workers (2008). Alternatively, copy number classification obtained from the PCA and LDF fitting models were used for analysis and differences in the copy numbers between control and IPF groups or between progressive and non-progressive groups were analysed by a 3x2 contingency table (χ^2 test with 2 degrees of freedom). Association of the >2 copy variant with disease susceptibility or progression was assessed by Fisher's exact test and the odds ratios were calculated.

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14. List of Abbreviations

2-BP	2-bromopalmitate
aCGH	array comparative genome hybridisation
ACPA	anti-citrullinated protein/ peptide antibodies
ADAM	a disintegrin and metalloproteinase
ADCC	antibody-dependent cellular cytotoxicity
AF	AlexaFluor™
AIP	acute interstitial pneumonitis
Amp	ampicillin
ANCA	anti-neutrophil cytoplasmic antibody
ANOVA	analysis of variance
APC	allophycocyanin
APS	anti-phospholipid syndrome
ATS	American Thoracic Society
BAI-1	brain-specific angiogenesis inhibitor 1
BAL	bronchoalveolar lavage
BCR	B-cell receptor
BL	Burkitt lymphoma
BPIP	bactericidal permeability-increasing protein
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
BxB	biotin - anti-biotin complex
C	complement
C2	C2-ceramide
C2-dh	C2-dihydroceramide
C4-S	chondroitin-4-sulfate
C6	C6-ceramide
CAP-37	cationic antimicrobial protein 37
CBA	cytometric bead array
CHL	cholesterol
CHO	Chinese Hamster Ovary
CHO:32	CD32a-transfected Chinese Hamster Ovary cells
CHX	cycloheximide
CI	confidence interval

CIDP	chronic inflammatory demyelinating polyneuropathy
CMFDA	*5*-chloromethylfluorescein diacetate
CMV	human cytomegalovirus
CNV	copy number variation
COP	cryptogenic organising pneumonia
cppt	central polypurine tract
CR	complement receptor
CRP	C-reactive protein
CTD	connective tissue disease
CXR	chest X ray
Cyto D	cytochalasin D
DAG	diacylglycerol
DC	dendritic cells
DDAO-SE	*7*-hydroxy-*9*H-(*1,3*-dichloro-*9,9*-dimethylacridin-*2*-one) succinimidyl ester
df	degrees of freedom
DHR123	dihydrorhodamine 123
DIP	desquamative interstitial pneumonia
DL_{CO}	diffusing capacity of the lung for carbon monoxide
DMB	3,3'-dimethoxybenzidine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DPLD	diffuse parenchymal lung disease
DRM	detergent-resistant membranes
ECCS	European Community for Coal and Steel
ECL	Enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ERS	European Respiratory Society
F	female
Fab	fragment, antibody binding
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FBS	foetal bovine serum
Fc	fragment crystallisable
FCS	foetal calf serum

FcγR	Fcγ receptor
FDC	follicular dendritic cells
FEV_1	forced expiratory volume in 1 sec
FI	fluorescence intensity
FITC	Fluorescein isothiocyanate
fMLP	formyl-methionyl-leucyl-phenylalanine
FPR	*N*-formylpeptide receptor
FSC	forward scatter (flow cytometry)
FVC	forced vital capacity
G-CSF	granulocyte colony-stimulating factor
Gas-6	growth arrest-specific protein 6
GATA-4	GATA-binding factor 4
GBS	Guillain-Barré syndrome
GDNF	Glial cell-derived neurotrophic factor
GM-CSF	granulocyte macrophage colony-stimulating factor
Go	goat
GPI	glycophosphatidylinositol
H_2O_2	hydrogen peroxide
Ha	hamster
HapMap	haplotype map
HBSS	Hanks' Balanced Salt Solution
Hck	haemopoietic cell kinase
hEF-1α	human elongation factor 1α
HEPES	*4*-(*2*-hydroxyethyl)-*1*-piperazineethanesulfonic acid
hHAIgG	human heat aggregated IgG
hi	heat inactivated
HIT	heparin-induced thrombocytopenia
HLA	human leukocyte antigen
HNA	human neutrophil antigen
HNE	human neutrophil elastase
HOCl	hypochlorous acid
hPGK	human phosphoglycerate kinase (promoter)
HPLC	high performance liquid chromatography
HRCT	high resolution computed tomography
HRP	Horseradish peroxidase
HTLV	human T-cell leukaemia virus

Hu	human
HX	histiocytosis X
IC	immune complex
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon- γ
Ig	immunoglobulin
IIP	idiopathic interstitial pneumonias
IL	interleukin
ILD	interstitial lung disease
IMDM	Iscove's Modified Dulbecco's Medium
IP	immunoprecipitation
IP_3	inositol triphosphate
IPF	idiopathic pulmonary fibrosis
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
ITP	idiopathic thrombocytopenia purpura
JNK	c-Jun N-terminal kinase
Jurkat:32	CD32a-transfected Jurkat cells
Kan	kanamycin
KCO	DL_{CO} corrected for lung volume
KIR	killer cell immunoglobulin-like receptor
LAM	lymphangioleiomyomatosis
LB	Lysogeny Broth
Lck	lymphocyte cell-specific protein-tyrosine kinase
LDF	linear discriminant function
LFA-1	lymphocyte function-associated antigen-1
LIP	lymphocytic interstitial pneumonia
LOX-1	lectin-like oxidised LDL receptor
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
LTR	long terminal repeat
M	male
mAb	monoclonal antibody
MAP	mitogen-activated protein
MBL	mannan-binding lectin
MCP-1	monocyte chemotactic protein-1

mcs	multiple cloning site
MEK	MAPK/ERK kinase
Mertk	Mer tyrosine kinase
MFG-E8	milk fat globule EGF-factor 8
MG	myasthenia gravis
MHC II	major histocompatibility complex class II
MMP	matrix metalloproteinase
Mo	mouse
Mono	monoclonal
MPO	myeloperoxidase
MWCO	molecular weight-cut-off
MβCD	methyl-β-cyclodextrin
Mϕ	macrophages
NA	neutrophil antigen
NAPDH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
NFAT	nuclear factor of activated T-cells
NGAL	neutrophil gelatinase-associated lipocalin
NHS	National Heath Service
NK	natural killer
NOS	nitric oxide synthase
NRAMP1	natural resistance-associated macrophage protein 1
NS	non significant
NSIP	non-specific interstitial pneumonia
Nϕ	neutrophil
OHO	hydroxyl radical
ONOO	peroxynitrite
OR	odds ratio
ORF	open reading frame
Ori	origin
P	phospho
$P(A–a)O_2$	difference between alveolar and arterial pressure
PAMP	pathogen-associated molecular pattern
pAn	polyadenylation (signal)
PaO_2	arterial oxygen tension
PBS	phosphate buffered saline

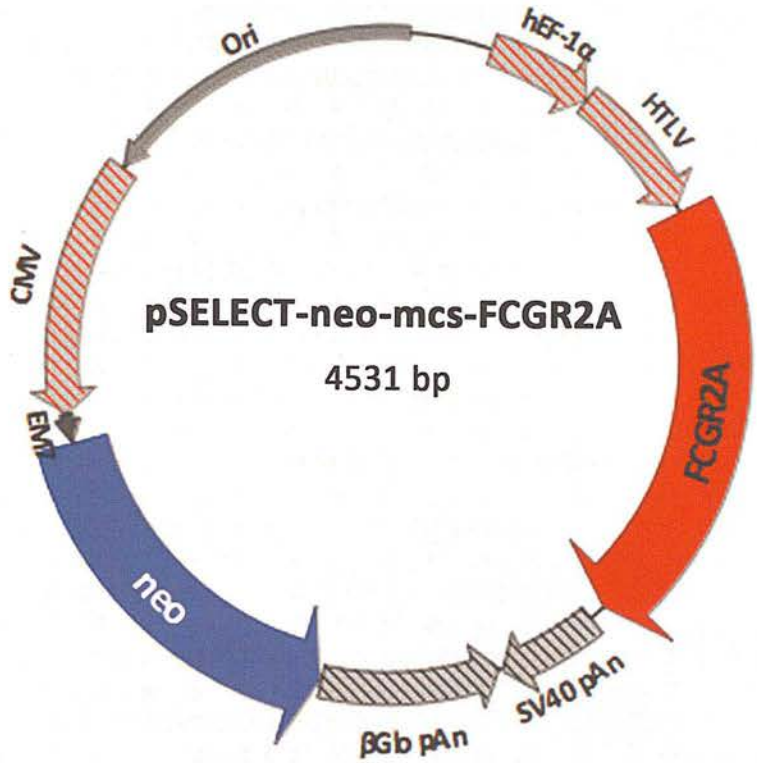
PCA	principal components analysis
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	propidium iodide
PI-PLC	phosphatidylinositol-specific phospholipase C
PI3K	phosphoinositide 3-kinase
pIgR	polymeric immunoglobulin receptor
PLCγ	phospholipase C γ
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocytes
PMSF	phenylmethanesulfonylfluoride
Poly	polyclonal
PrK	proteinase K
PS	phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PtdIns(3,4,5)P_3	phosphatidylinositol 3,4,5-triphosphate
PtdIns(4,5)P_2	phosphatidylinositol 4,5-biphosphate
Puro	puromycin resistance gene
pY	tyrosine phosphorylation
R-PE	R-Phycoerythrin
RA	rheumatoid arthritis
Ra	rabbit
RB-ILD	respiratory bronchiolitis-associated interstitial lung disease
RF	rheumatic fever
RGD	arginine-glycine-aspartic acid (peptide motif)
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
RP-HPLC	reverse phase high performance liquid chromatography
RRE	rev response element
SAP	serum amyloid protein
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHC	Src homology-containing protein
SHIP	SH2 domain-containing Inositol 5-phosphatase
SHP2	SH2 domain-containing phosphatase
shRNA	short hairpin RNA

SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SP	surfactant protein
SRA	scavenger receptor A
SSC	side scatter (flow cytometry)
SV40	Simian virus 40
Syk	spleen tyrosine kinase
TB	Terrific Broth
TBE	Tris Borate EDTA
TCR	T-cell receptor
TGF-β	transforming growth factor-β
Th	T helper
TIM-4	T-cell immunoglobulin and mucin domain-containing protein 4
TLC	total lung capacity
TLR	Toll-like receptors
TNF-α	tumour necrosis factor-α
TSP	thrombospondin
Txn	transcription terminator sequences
UV	ultra violet
VC	vital capacity
WG	Wegener's granulomatosis
WGTP	Whole Genome TilePath
WT	wild type
YY-1	Yin-Yang 1
ZAP-70	ζ chain protein kinase 70
α-CD	α-cyclodextrin
α-SMA	α-smooth muscle actin
β_2-GPI	β_2-glycoprotein-I
βGlo	human β globin gene
ψ	ψ sequence - RNA packaging signal

15. Appendices

15.1 Plasmid Maps and Sequences

15.1.1. pSELECT-neo-mcs-FCGR2A



Ori: *E.coli* origin of replication; **CMV**: human cytomegalovirus immediate-early gene 1 promoter enhancer; **EM7**: bacterial *E.coli* constitutive promoter; ***neo***: *neo* gene conferring resistance to kanamycin in *E.coli* and to G418 in mammalian cells; **βGlo pAn**: human β-globin 3'UTR and polyadenylation sequence; **hEF-1α**: human elongation factor 1α (EF-1α) core promoter; **HTLV**: the R segment and part of the U5 sequence (R-U5') of the human T-cell leukaemia virus (HTLV) type 1 long terminal repeat (LTR); ***FCGR2A***: *FCGR2A* ORF; **SV40 pAn**: Simian virus 40 late polyadenylation signal.

```
   1 TCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGGCTAGTTAATTAACATTTAA
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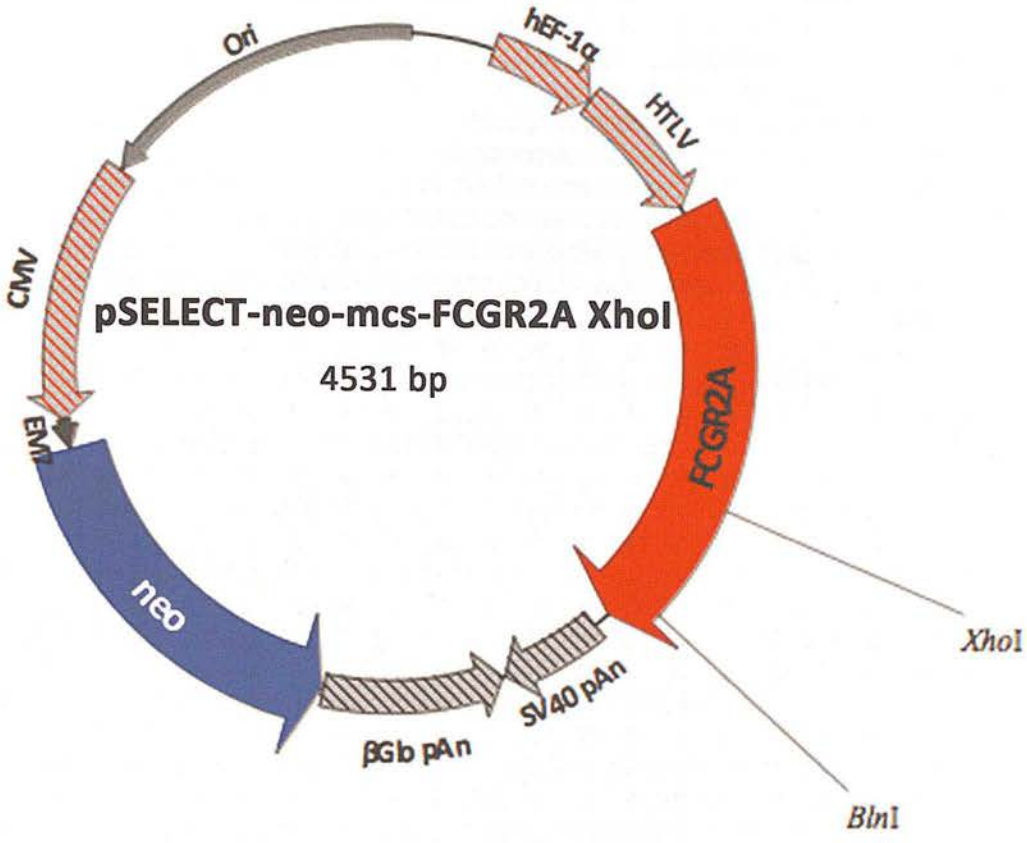

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```
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     W  L  L  Q  P  L  T  V  L  L  L  L  A  S  A  D  S  Q  A  A
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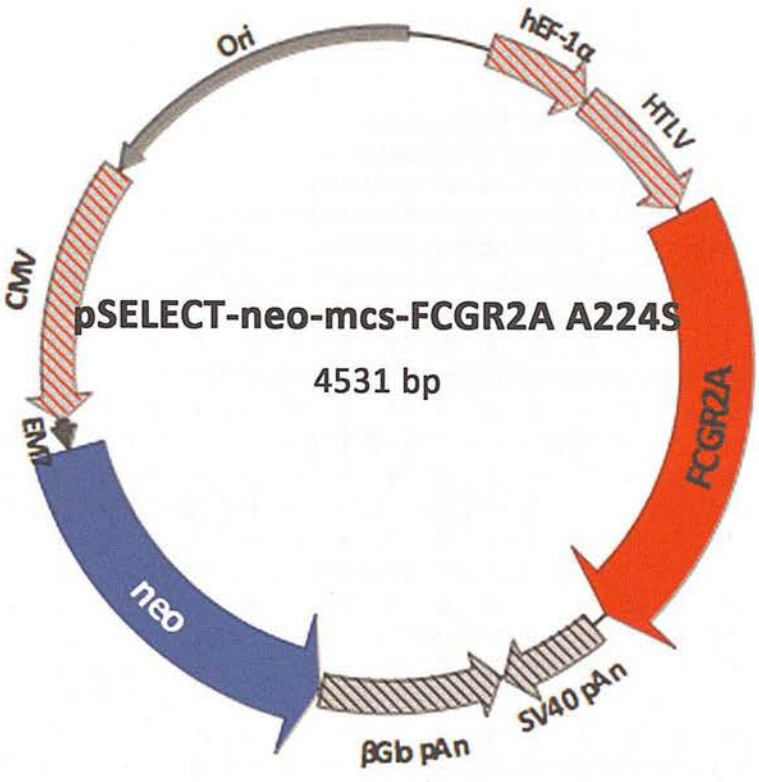

Ori: *E.coli* origin of replication; **CMV**: human cytomegalovirus immediate-early gene 1 promoter enhancer; **EM7**: bacterial *E.coli* constitutive promoter; ***neo***: *neo* gene conferring resistance to kanamycin in *E.coli* and to G418 in mammalian cells; **βGlo pAn**: human β-globin 3'UTR and polyadenylation sequence; **hEF-1α**: human elongation factor 1α (EF-1α) core promoter; **HTLV**: the R segment and part of the U5 sequence (R-U5') of the human T-cell leukaemia virus (HTLV) type 1 long terminal repeat (LTR); ***FCGR2A***: *FCGR2A* ORF; **SV40 pAn**: Simian virus 40 late polyadenylation signal.

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 421 GTAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAACACAGCTGAAGCTT
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 541 CGGTTGAGTCGCGTTCTGCCGCCTCCCGCCTGTGGTGCCTCCTGAACTGCGTCCGCCGTC
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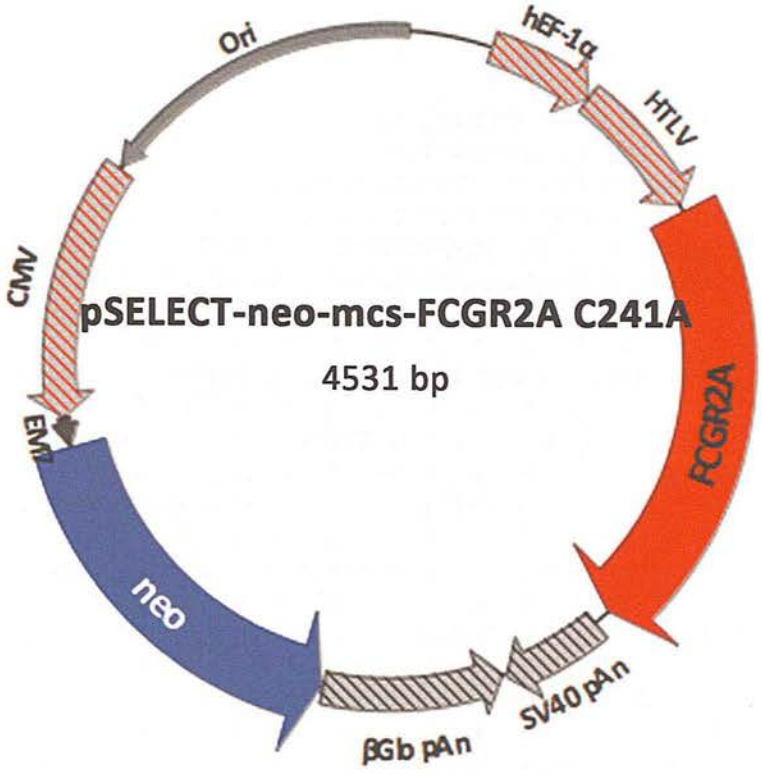

Ori: *E.coli* origin of replication; **CMV**: human cytomegalovirus immediate-early gene 1 promoter enhancer; **EM7**: bacterial *E.coli* constitutive promoter; ***neo***: *neo* gene conferring resistance to kanamycin in *E.coli* and to G418 in mammalian cells; **βGlo pAn**: human β-globin 3'UTR and polyadenylation sequence; **hEF-1α**: human elongation factor 1α (EF-1α) core promoter; **HTLV**: the R segment and part of the U5 sequence (R-U5') of the human T-cell leukaemia virus (HTLV) type 1 long terminal repeat (LTR); ***FCGR2A***: *FCGR2A* ORF; **SV40 pAn**: Simian virus 40 late polyadenylation signal.

```
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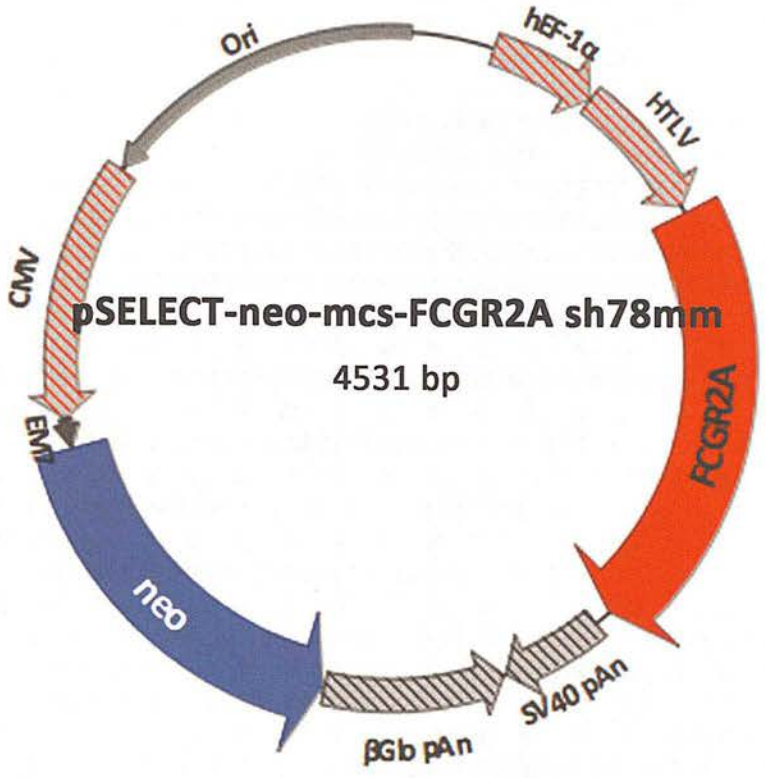

Ori: *E.coli* origin of replication; **CMV**: human cytomegalovirus immediate-early gene 1 promoter enhancer; **EM7**: bacterial *E.coli* constitutive promoter; ***neo***: *neo* gene conferring resistance to kanamycin in *E.coli* and to G418 in mammalian cells; **βGlo pAn**: human β-globin 3'UTR and polyadenylation sequence; **hEF-1α**: human elongation factor 1α (EF-1α) core promoter; **HTLV**: the R segment and part of the U5 sequence (R-U5') of the human T-cell leukaemia virus (HTLV) type 1 long terminal repeat (LTR); ***FCGR2A***: *FCGR2A* ORF; **SV40 pAn**: Simian virus 40 late polyadenylation signal.

```
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 241 CGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCCACAGTCCCCGAGAAGTTGGGG
 301 GGAGGGGTCGGCAATTGAACGGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAAGT
 361 GATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCA
 421 GTAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAACACAGCTGAAGCTT
 481 CGAGGGGCTCGCATCTCTCCTTCACGCGCCCGCCGCCCTACCTGAGGCCGCCATCCACGC
 541 CGGTTGAGTCGCGTTCTGCCGCCTCCCGCCTGTGGTGCCTCCTGAACTGCGTCCGCCGTC
 601 TAGGTAAGTTTAAAGCTCAGGTCGAGACCGGGCCTTTGTCCGGCGCTCCCTTGGAGCCTA
 661 CCTAGACTCAGCCGGCTCTCCACGCTTTGCCTGACCCTGCTTGCTCAACTCTACGTCTTT
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 781 CACCGGTCATCATGACTATGGAGACCCAAATGTCTCAGAATGTATGTCCCAGAAACCTGT
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 841 GGCTGCTTCAACCATTGACAGTTTTGCTGCTGCTGGCTTCTGCAGACAGTCAAGCTGCAG
     W  L  L  Q  P  L  T  V  L  L  L  L  A  S  A  D  S  Q  A  A
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     L  T  V  L  S  E  W  L  V  L  Q  T  P  H  L  E  F  Q  E  G
1201 AAACCATCATGCTGAGGTGCCACAGCTGGAAGGACAAGCCTCTGGTCAAGGTCACATTCT
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2401 CGAGCTTCTAGCTTTAGAAGAACTCATCAAGAAGTCTGTAGAAGGCAATTCTCTGGGAGT
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2461 CAGGGGCTGCAATGCCATAGAGCACTAGGAACCTGTCTGCCCACTCTCCCCCTAGCTCTT
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       G  H  T  V  V  L  D  E  G  D  P  M  S  A  K  L  R  A  F  L
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3481 AACCGCTATCCACGCCCATTGATGTACTGCCAAAACCGCATCATCATGGTAATAGCGATG
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3601 TGCCAGGCGGGCCATTTACCGTCATTGACGTCAATAGGGGGCGTACTTGGCATATGATAC
3661 ACTTGATGTACTGCCAAGTGGGCAGTTTACCGTAAATACTCCACCCATTGACGTCAATGG
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4081 CGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCG
4141 TTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTAT
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4441 GCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAG
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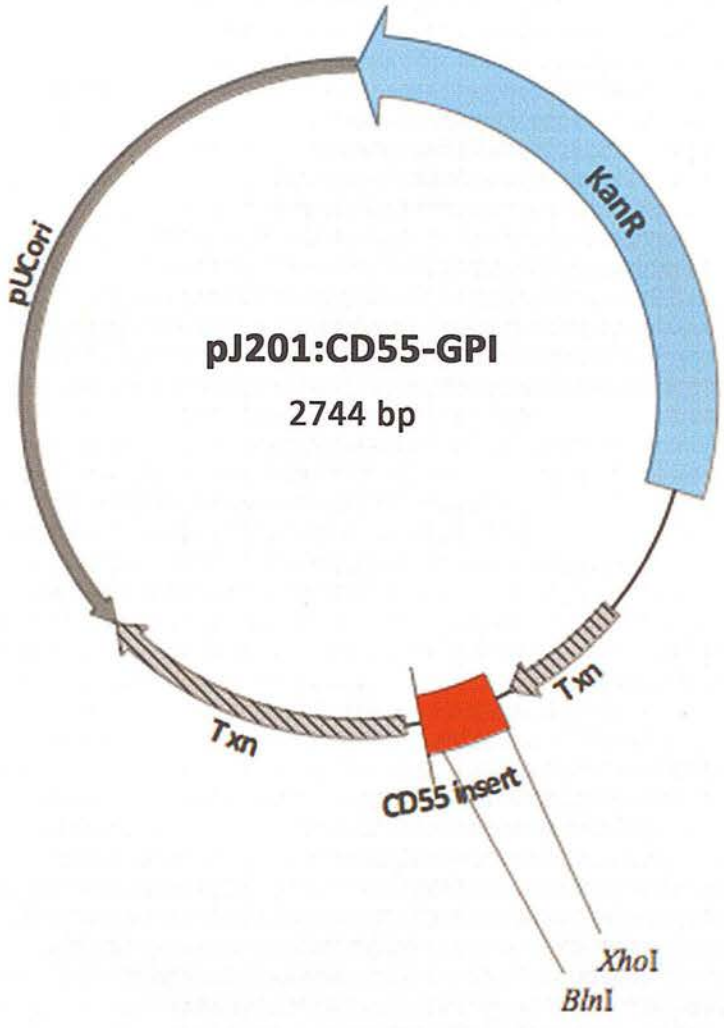
15.1.6. pJ201:CD55-GPI



pUCori: *E.coli* origin of replication from a pUC vector; **KanR**: kanamycin resistance gene; **CD55 insert**: GPI-consensus sequence from the human *CD55* gene; **Txn**: bacterial transcription terminator sequences.

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1051 CCGTAGCGCCGATGGTAGTGTGGGGACTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACG
1121 AAAGGCTCAGTCGAAAGACTGGGCCTTTCGCCCGGGCTAATTAGGGGGTGTCGCCCTTTGAACCCTCGAG
1191 TGGTACTACCCGTCTTCTATCTGGGCACACGTGTTTCACGTTGACAGGTTTGCTTGGGACGCTAGTAACC
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2031 TTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCC
2101 GACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTG
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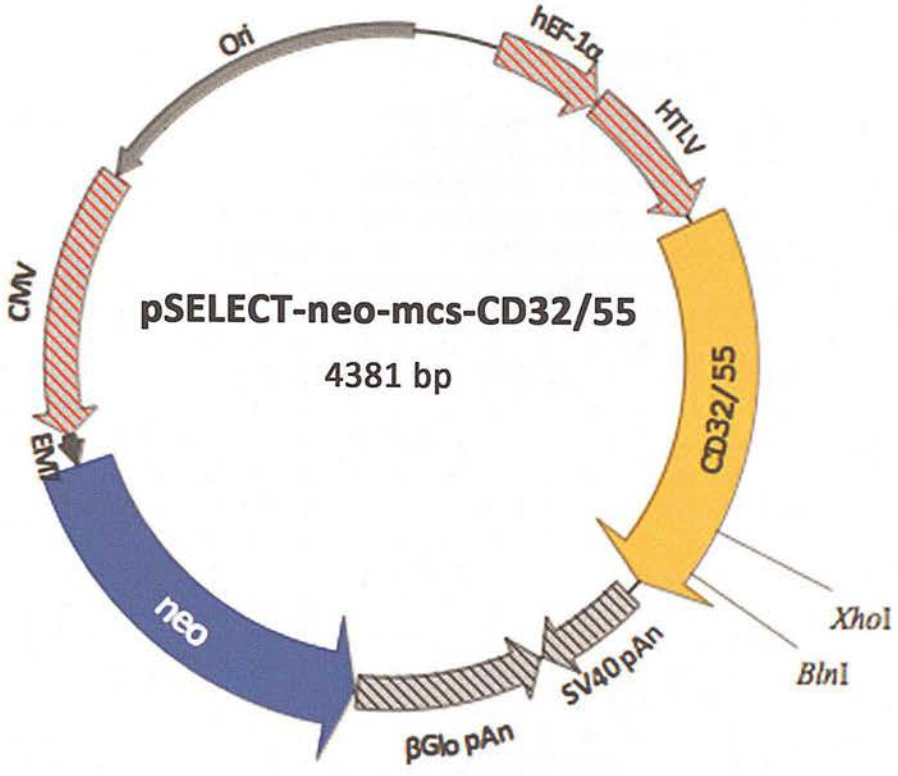
15.1.7. pSELECT-neo-mcs-CD32/55



Ori: *E.coli* origin of replication; **CMV**: human cytomegalovirus immediate-early gene 1 promoter enhancer; **EM7**: bacterial *E.coli* constitutive promoter; ***neo***: *neo* gene conferring resistance to kanamycin in *E.coli* and to G418 in mammalian cells; **βGlo pAn**: human β-globin 3'UTR and polyadenylation sequence; **hEF-1α**: human elongation factor 1α (EF-1α) core promoter; **HTLV**: the R segment and part of the U5 sequence (R-U5') of the human T-cell leukaemia virus (HTLV) type 1 long terminal repeat (LTR); **CD32/55**: *CD32* ORF comprising the *CD55* GPI consensus sequence; **SV40 pAn**: Simian virus 40 late polyadenylation signal.

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 301 GGAGGGGTCGGCAATTGAACGGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAAGT
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 421 GTAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAACACAGCTGAAGCTT
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 541 CGGTTGAGTCGCGTTCTGCCGCCTCCCGCCTGTGGTGCCTCCTGAACTGCGTCCGCCGTC
 601 TAGGTAAGTTTAAAGCTCAGGTCGAGACCGGGCCTTTGTCCGGCGCTCCCTTGGAGCCTA
 661 CCTAGACTCAGCCGGCTCTCCACGCTTTGCCTGACCCTGCTTGCTCAACTCTACGTCTTT
 721 GTTTCGTTTTCTGTTCTGCGCCGTTACAGATCCAAGCTGTGACCGGCGCCTACCTGAGAT
 781 CACCGGTCATCATGACTATGGAGACCCAAATGTCTCAGAATGTATGTCCCAGAAACCTGT
                 M  T  M  E  T  Q  M  S  Q  N  V  C  P  R  N  L
 841 GGCTGCTTCAACCATTGACAGTTTTGCTGCTGCTGGCTTCTGCAGACAGTCAAGCTGCAG
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      P  M  S  A  K  L  R  A  F  L  E  A  P  A  L  G  Q  H  E  E
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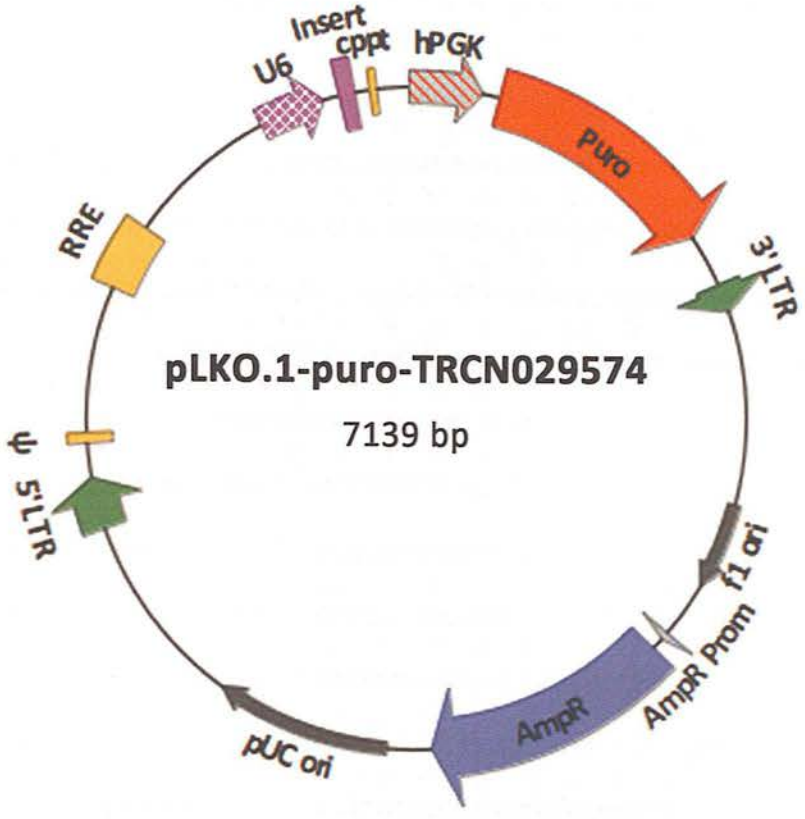

U6: U6 promoter; **insert**: shRNA-encoding insert sequence; **cppt**: central polypurine tract; **hPGK**: human phosphoglycerate kinase eukaryotic promoter; **Puro**: puromycin resistance gene for mammalian selection; **3' LTR**: 3' self inactivating long terminal repeat; **f1 ori**: *E.coli* f1 origin of replication; **AmpR Prom**: bacterial constitutive promoter for the ampicillin resistance gene; **AmpR**: ampicillin resistance gene for bacterial selection; **pUC ori**: pUC-based *E.coli* origin of replication; **5' LTR**: L' long terminal repeat; **Ψ**: ψ sequence - RNA packaging signal; **RRE**: *Rev* response element.

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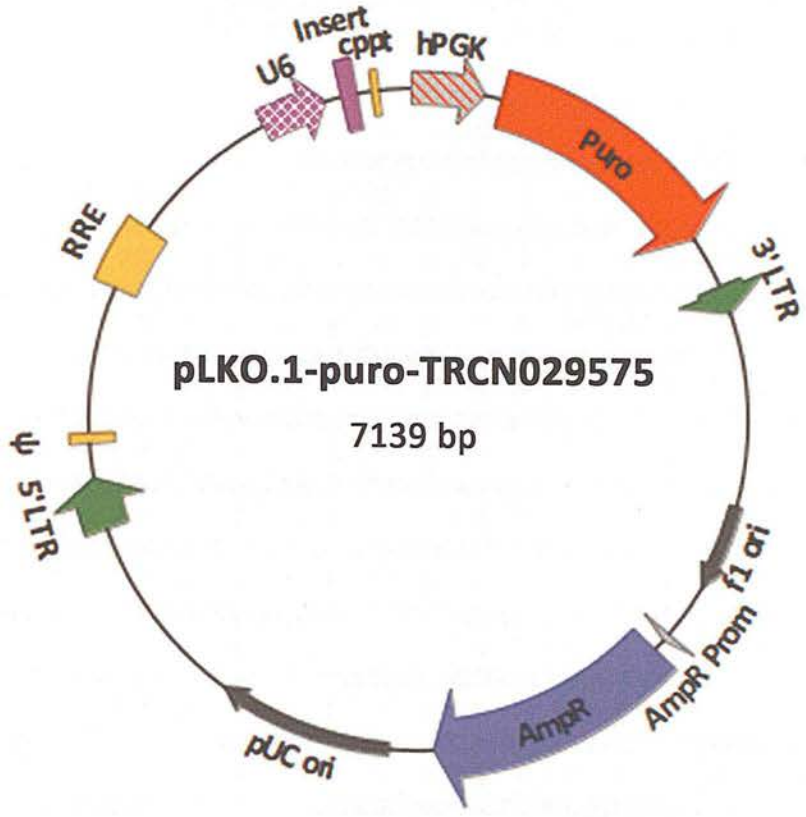

U6: U6 promoter; **insert**: shRNA-encoding insert sequence; **cppt**: central polypurine tract; **hPGK**: human phosphoglycerate kinase eukaryotic promoter; **Puro**: puromycin resistance gene for mammalian selection; **3' LTR**: 3' self inactivating long terminal repeat; **f1 ori**: *E.coli* f1 origin of replication; **AmpR Prom**: bacterial constitutive promoter for the ampicillin resistance gene; **AmpR**: ampicillin resistance gene for bacterial selection; **pUC ori**: pUC-based *E.coli* origin of replication; **5' LTR**: L' long terminal repeat; **Ψ**: ψ sequence - RNA packaging signal; **RRE**: *Rev* response element.

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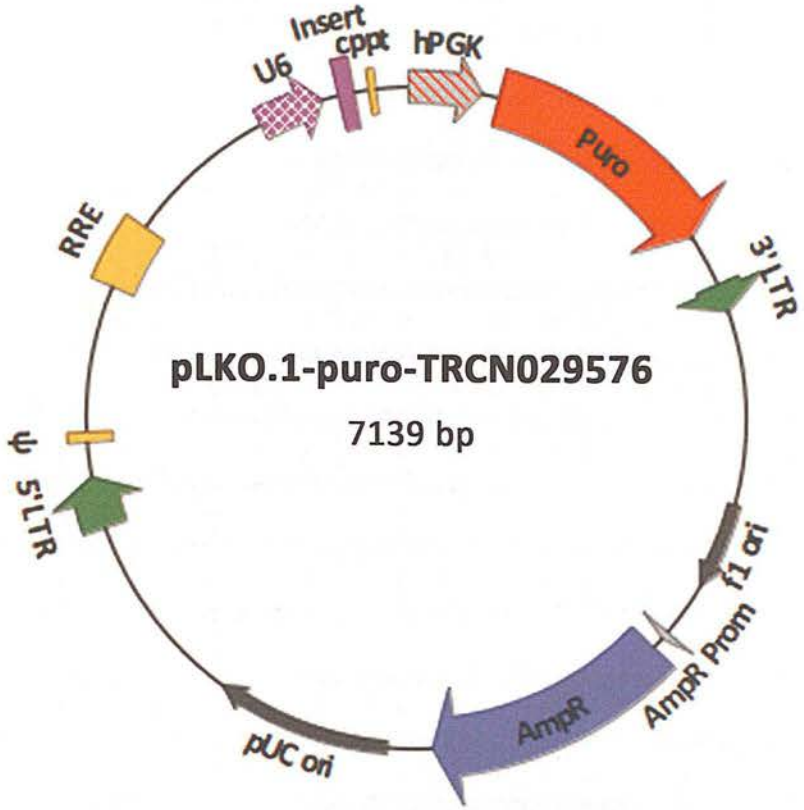

U6: U6 promoter; **insert**: shRNA-encoding insert sequence; **cppt**: central polypurine tract; **hPGK**: human phosphoglycerate kinase eukaryotic promoter; **Puro**: puromycin resistance gene for mammalian selection; **3' LTR**: 3' self inactivating long terminal repeat; **f1 ori**: *E.coli* f1 origin of replication; **AmpR Prom**: bacterial constitutive promoter for the ampicillin resistance gene; **AmpR**: ampicillin resistance gene for bacterial selection; **pUC ori**: pUC-based *E.coli* origin of replication; **5' LTR**: L' long terminal repeat; **ψ**: ψ sequence - RNA packaging signal; **RRE**: *Rev* response element.

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       R  E  G  A  V  R  E  A  G  C  G  A  V  V  W  A  L  F  L  P
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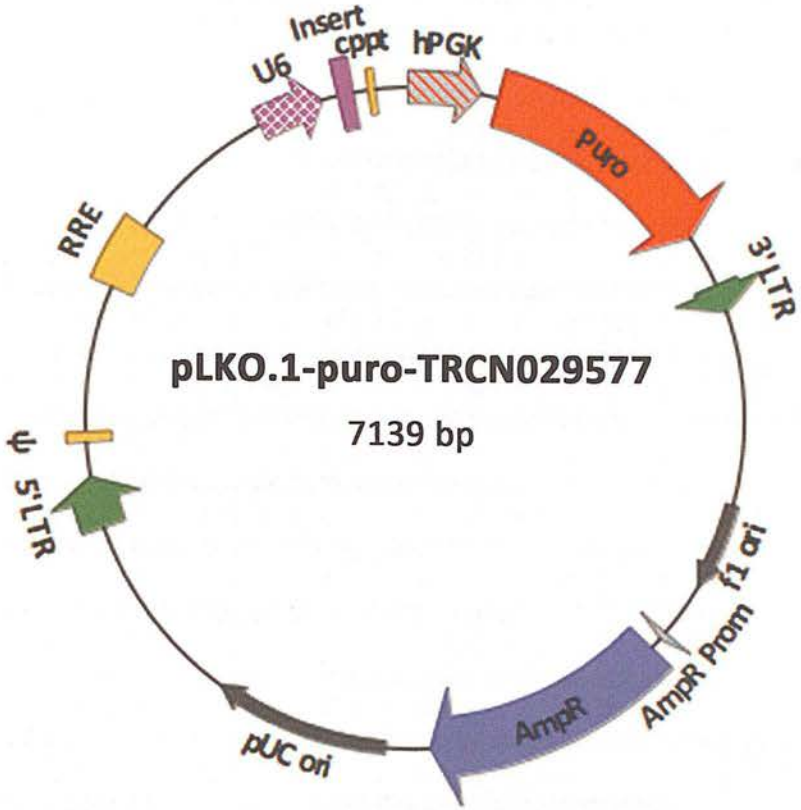

U6: U6 promoter; **insert**: shRNA-encoding insert sequence; **cppt**: central polypurine tract; **hPGK**: human phosphoglycerate kinase eukaryotic promoter; **Puro**: puromycin resistance gene for mammalian selection; **3' LTR**: 3' self inactivating long terminal repeat; **f1 ori**: *E.coli* f1 origin of replication; **AmpR Prom**: bacterial constitutive promoter for the ampicillin resistance gene; **AmpR**: ampicillin resistance gene for bacterial selection; **pUC ori**: pUC-based *E.coli* origin of replication; **5' LTR**: L' long terminal repeat; **ψ**: ψ sequence - RNA packaging signal; **RRE**: *Rev* response element.

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      P  E  G  P  R  T  W  C  M  T  R  K  P  G  A  *
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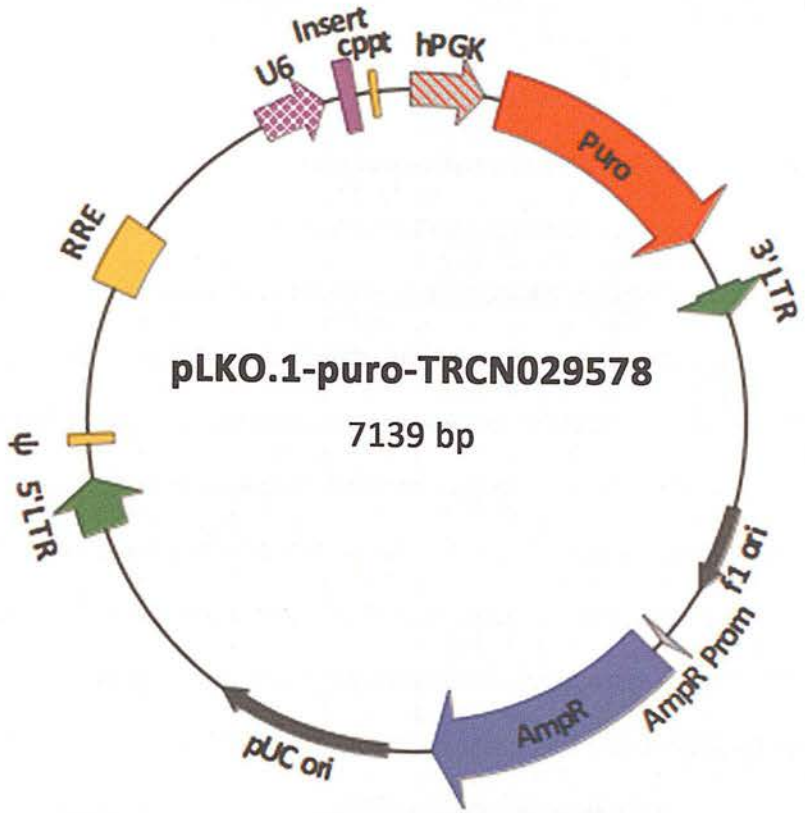

U6: U6 promoter; **insert**: shRNA-encoding insert sequence; **cppt**: central polypurine tract; **hPGK**: human phosphoglycerate kinase eukaryotic promoter; **Puro**: puromycin resistance gene for mammalian selection; **3' LTR**: 3' self inactivating long terminal repeat; **f1 ori**: *E.coli* f1 origin of replication; **AmpR Prom**: bacterial constitutive promoter for the ampicillin resistance gene; **AmpR**: ampicillin resistance gene for bacterial selection; **pUC ori**: pUC-based *E.coli* origin of replication; **5' LTR**: L' long terminal repeat; **ψ**: ψ sequence - RNA packaging signal; **RRE**: *Rev* response element.

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       R  P  R  W  A  V  A  N  S  G  C  S  A  G  R  A  E  S  S  G
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       R  E  G  A  V  R  E  A  G  C  G  A  V  V  W  A  L  F  L  P
 181 CCGCCCCTAAGTCGGGAAGGTTCCTTGCGGTTCGCGGCGTGCCGGACGTGACAAACGGAA
       A  R  C  S  A  F  C  K  P  P  E  R  T  S  A  V  G  S  L  V
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       K  P  T  V  R  L  A  T  R  D  D  V  P  R  A  V  R  T  L  A
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6421 CCCACCTCCCAACCCCGAGGGGACCCGACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAG
6481 AGAGAGACAGAGACAGATCCATTCGATTAGTGAACGGATCTCGACGGTATCGATCACGAG
6541 ACTAGCCTCGAGCGGCCGCCCCCTTCACCGAGGGCCTATTTCCCATGATTCCTTCATATT
6601 TGCATATACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAA
6661 AGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTT
6721 TAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATT
6781 TCTTGGCTTTATATATCTTGTGGAAAGGACGAAACA**CCGGGAAGAAACCAACAATGACTA**
6841 **TCTCGAGATAGTCATTGTTGGTTTCTTCTTTTT**AATCTGTCAGCATCTGGGTCATTCATA
6901 ATAATAATATCTGCATCATGTTTAATACCATATTCAAGCGGTATTTTTCATGCAGGATCA
6961 AATTCTGGATTTTAAAAGAAAAGGGGGGATTGGGGGGTACAGTGCAGGGGAAAGAATAGT
7021 AGACATAATAGCAACAGACATACAAACTAAAGAATTACAAAAACAAATTACAAAAATTCA
7081 AAATTTTCGGGTTTATTACAGGGACAGCAGAGATCCACTTTGGCCGCGGCTCGAGGGGG

15.2 Posterior Probability and Copy Number Assignment

15.2.1. PCA-transformed Data

A. Control

Trait	*FCGR3B:CD36* Raw Ratio	P1	P2	P3	Copy Number
Control	0.9675	0.000	0.995	0.005	2
Control	1.1136	0.000	0.978	0.022	2
Control	1.0466	0.000	0.990	0.010	2
Control	1.1669	0.000	0.954	0.046	2
Control	1.1915	0.000	0.932	0.068	2
Control	1.1216	0.000	0.976	0.024	2
Control	1.1840	0.000	0.940	0.060	2
Control	0.4641	1.000	0.000	0.000	1
Control	1.0596	0.000	0.989	0.011	2
Control	1.0641	0.000	0.988	0.012	2
Control	0.9536	0.000	0.996	0.004	2
Control	1.4091	0.000	0.134	0.866	3
Control	1.2356	0.000	0.865	0.135	2
Control	1.5345	0.000	0.005	0.995	3
Control	1.1130	0.000	0.978	0.022	2
Control	1.2173	0.000	0.899	0.101	2
Control	1.0863	0.000	0.985	0.015	2
Control	1.0292	0.000	0.992	0.008	2
Control	1.6498	0.000	0.000	1.000	3
Control	1.1051	0.000	0.980	0.020	2
Control	1.4383	0.000	0.069	0.931	3
Control	1.1612	0.000	0.957	0.043	2
Control	2.0798	0.000	0.000	1.000	3
Control	1.0612	0.000	0.989	0.011	2
Control	1.2316	0.000	0.874	0.126	2
Control	1.1481	0.000	0.965	0.035	2
Control	1.6086	0.000	0.001	0.999	3
Control	0.5232	0.999	0.001	0.000	1
Control	1.5153	0.000	0.009	0.991	3
Control	1.0976	0.000	0.982	0.018	2
Control	1.2431	0.000	0.848	0.152	2
Control	0.6179	0.970	0.030	0.000	1
Control	0.8911	0.000	0.997	0.003	2
Control	1.8126	0.000	0.000	1.000	3
Control	1.0479	0.000	0.990	0.010	2
Control	1.6222	0.000	0.000	1.000	3
Control	1.0837	0.000	0.985	0.015	2
Control	0.5555	0.997	0.003	0.000	1
Control	0.7793	0.008	0.990	0.002	2
Control	1.0002	0.000	0.994	0.006	2

Trait	*FCGR3B:CD36* Raw Ratio	P1	P2	P3	Copy Number
Control	0.9995	0.000	0.994	0.006	2
Control	0.8326	0.000	0.997	0.002	2
Control	1.0619	0.000	0.988	0.012	2
Control	1.0054	0.000	0.994	0.006	2
Control	0.9738	0.000	0.995	0.005	2
Control	1.0524	0.000	0.990	0.010	2
Control	1.0869	0.000	0.984	0.016	2
Control	1.0464	0.000	0.990	0.010	2
Control	1.3716	0.000	0.276	0.724	3
Control	0.9999	0.000	0.994	0.006	2
Control	0.9464	0.000	0.996	0.004	2
Control	1.1635	0.000	0.956	0.044	2
Control	2.0339	0.000	0.000	1.000	3
Control	1.4495	0.000	0.052	0.948	3
Control	1.0779	0.000	0.986	0.014	2
Control	1.0276	0.000	0.992	0.008	2
Control	1.5403	0.000	0.004	0.996	3
Control	0.9003	0.000	0.997	0.003	2
Control	1.0512	0.000	0.990	0.010	2
Control	1.0740	0.000	0.987	0.013	2
Control	1.4166	0.000	0.113	0.887	3
Control	1.1193	0.000	0.976	0.024	2
Control	0.6000	0.986	0.014	0.000	1
Control	1.5125	0.000	0.010	0.990	3
Control	1.2728	0.000	0.761	0.239	2
Control	1.0324	0.000	0.992	0.008	2
Control	1.0751	0.000	0.987	0.013	2
Control	0.7693	0.015	0.983	0.002	2
Control	1.6080	0.000	0.001	0.999	3
Control	0.6287	0.953	0.047	0.000	1
Control	0.8811	0.000	0.997	0.003	2
Control	0.9627	0.000	0.996	0.004	2
Control	1.0664	0.000	0.988	0.012	2
Control	1.0255	0.000	0.992	0.008	2
Control	0.9619	0.000	0.996	0.004	2
Control	0.4475	1.000	0.000	0.000	1
Control	0.9297	0.000	0.996	0.004	2
Control	0.5917	0.990	0.010	0.000	1
Control	1.4929	0.000	0.017	0.983	3
Control	0.3965	1.000	0.000	0.000	1
Control	1.1582	0.000	0.959	0.041	2
Control	0.7965	0.003	0.995	0.002	2
Control	1.0518	0.000	0.990	0.010	2
Control	0.9570	0.000	0.996	0.004	2
Control	0.9766	0.000	0.995	0.005	2
Control	0.8970	0.000	0.997	0.003	2
Control	1.0066	0.000	0.994	0.006	2
Control	1.0621	0.000	0.988	0.012	2
Control	1.0332	0.000	0.992	0.008	2

Trait	*FCGR3B:CD36* Raw Ratio	P1	P2	P3	Copy Number
Control	0.9574	0.000	0.996	0.004	2
Control	0.4569	1.000	0.000	0.000	1
Control	1.1141	0.000	0.978	0.022	2
Control	0.9340	0.000	0.996	0.004	2
Control	0.4811	1.000	0.000	0.000	1
Control	1.7737	0.000	0.000	1.000	3
Control	1.6154	0.000	0.000	1.000	3
Control	1.0987	0.000	0.982	0.018	2
Control	1.1100	0.000	0.979	0.021	2
Control	0.9499	0.000	0.996	0.004	2
Control	1.1003	0.000	0.982	0.018	2
Control	1.0909	0.000	0.984	0.016	2
Control	1.1700	0.000	0.951	0.049	2
Control	0.4865	1.000	0.000	0.000	1
Control	0.9442	0.000	0.996	0.004	2
Control	1.0979	0.000	0.982	0.018	2
Control	0.1143	1.000	0.000	0.000	1
Control	1.0216	0.000	0.992	0.008	2
Control	1.0014	0.000	0.994	0.006	2
Control	0.4864	1.000	0.000	0.000	1
Control	1.0851	0.000	0.985	0.015	2
Control	1.1107	0.000	0.979	0.021	2
Control	0.5483	0.998	0.002	0.000	1
Control	1.0926	0.000	0.983	0.017	2
Control	0.4371	1.000	0.000	0.000	1
Control	1.0294	0.000	0.992	0.008	2
Control	1.5431	0.000	0.004	0.996	3
Control	0.8870	0.000	0.997	0.003	2
Control	1.5951	0.000	0.001	0.999	3
Control	1.6733	0.000	0.000	1.000	3
Control	1.1494	0.000	0.964	0.036	2
Control	1.1069	0.000	0.980	0.020	2
Control	2.1450	0.000	0.000	1.000	3
Control	1.0820	0.000	0.985	0.015	2
Control	1.5047	0.000	0.012	0.988	3
Control	0.5723	0.995	0.005	0.000	1
Control	1.1392	0.000	0.969	0.031	2
Control	1.0351	0.000	0.991	0.009	2
Control	1.6399	0.000	0.000	1.000	3
Control	1.1629	0.000	0.956	0.044	2
Control	1.1713	0.000	0.950	0.050	2
Control	1.5265	0.000	0.006	0.994	3
Control	1.0155	0.000	0.993	0.007	2
Control	1.2106	0.000	0.909	0.091	2
Control	1.1235	0.000	0.975	0.025	2
Control	1.1898	0.000	0.934	0.066	2
Control	2.1985	0.000	0.000	1.000	3
Control	1.1649	0.000	0.955	0.045	2
Control	0.7908	0.004	0.994	0.002	2

Trait	*FCGR3B:CD36* Raw Ratio	P1	P2	P3	Copy Number
Control	1.2374	0.000	0.861	0.139	2
Control	0.8664	0.000	0.997	0.002	2
Control	1.6999	0.000	0.000	1.000	3
Control	0.9503	0.000	0.996	0.004	2
Control	1.1712	0.000	0.950	0.050	2
Control	1.1835	0.000	0.940	0.060	2
Control	1.1094	0.000	0.979	0.021	2
Control	1.6298	0.000	0.000	1.000	3
Control	0.9602	0.000	0.996	0.004	2
Control	1.1193	0.000	0.976	0.024	2
Control	1.1977	0.000	0.926	0.074	2
Control	1.2232	0.000	0.889	0.111	2
Control	0.7059	0.332	0.666	0.001	2
Control	0.6585	0.838	0.162	0.000	1
Control	1.1769	0.000	0.946	0.054	2
Control	1.1293	0.000	0.973	0.027	2
Control	1.0575	0.000	0.989	0.011	2
Control	1.1321	0.000	0.972	0.028	2
Control	0.8824	0.000	0.997	0.003	2
Control	1.1446	0.000	0.966	0.034	2
Control	1.0170	0.000	0.993	0.007	2
Control	1.1387	0.000	0.969	0.031	2
Control	1.0670	0.000	0.988	0.012	2
Control	1.6784	0.000	0.000	1.000	3
Control	1.1555	0.000	0.961	0.039	2
Control	0.9733	0.000	0.995	0.005	2
Control	0.8573	0.000	0.998	0.002	2
Control	1.0854	0.000	0.985	0.015	2
Control	1.0535	0.000	0.989	0.011	2
Control	0.4852	1.000	0.000	0.000	1
Control	0.9983	0.000	0.994	0.006	2
Control	1.1105	0.000	0.979	0.021	2
Control	1.5186	0.000	0.008	0.992	3
Control	0.6881	0.552	0.447	0.001	1
Control	0.9880	0.000	0.995	0.005	2
Control	1.0191	0.000	0.993	0.007	2
Control	0.9526	0.000	0.996	0.004	2
Control	1.0391	0.000	0.991	0.009	2
Control	1.6512	0.000	0.000	1.000	3
Control	1.0940	0.000	0.983	0.017	2
Control	1.4911	0.000	0.017	0.983	3
Control	1.0378	0.000	0.991	0.009	2
Control	1.0900	0.000	0.984	0.016	2
Control	1.0064	0.000	0.994	0.006	2
Control	0.7814	0.007	0.991	0.002	2
Control	1.1613	0.000	0.957	0.043	2
Control	0.5986	0.986	0.014	0.000	1
Control	0.5771	0.994	0.006	0.000	1
Control	1.1817	0.000	0.942	0.058	2

Trait	*FCGR3B:CD36* Raw Ratio	P1	P2	P3	Copy Number
Control	1.3258	0.000	0.516	0.484	2
Control	1.1159	0.000	0.977	0.023	2
Control	1.0780	0.000	0.986	0.014	2
Control	1.1872	0.000	0.937	0.063	2
Control	1.0300	0.000	0.992	0.008	2
Control	1.2041	0.000	0.918	0.082	2
Control	0.9998	0.000	0.994	0.006	2
Control	1.0108	0.000	0.993	0.007	2
Control	1.5874	0.000	0.001	0.999	3
Control	1.6637	0.000	0.000	1.000	3
Control	1.5504	0.000	0.003	0.997	3
Control	0.6416	0.919	0.081	0.000	1
Control	1.4019	0.000	0.155	0.845	3
Control	1.1238	0.000	0.975	0.025	2
Control	1.0823	0.000	0.985	0.015	2
Control	1.1489	0.000	0.964	0.036	2
Control	1.3652	0.000	0.306	0.694	3
Control	1.1848	0.000	0.939	0.061	2
Control	1.4974	0.000	0.015	0.985	3
Control	1.3872	0.000	0.209	0.791	3
Control	1.1671	0.000	0.953	0.047	2
Control	1.1276	0.000	0.974	0.026	2
Control	1.3251	0.000	0.520	0.480	2
Control	0.8300	0.000	0.997	0.002	2
Control	1.0681	0.000	0.988	0.012	2
Control	1.1477	0.000	0.965	0.035	2
Control	1.1917	0.000	0.932	0.068	2
Control	0.4220	1.000	0.000	0.000	1
Control	1.1296	0.000	0.973	0.027	2
Control	1.3845	0.000	0.219	0.781	3
Control	1.1249	0.000	0.974	0.026	2
Control	0.9329	0.000	0.996	0.004	2
Control	1.0084	0.000	0.993	0.007	2
Control	1.9476	0.000	0.000	1.000	3

Abbreviations: P1: probability for 1 copy; P2: probability for 2 copies; P3: probability for 3 copies; *FCGR3B*:*CD36* ratio: ratio of *FCGR3B*- to *CD36*-specific qPCR amplification.

B. IPF

Trait	*FCGR3B:CD36* Raw Ratio	P1	P2	P3	Copy Number
IPF	2.1711	0.000	0.000	1.000	3
IPF	1.3968	0.000	0.270	0.730	3
IPF	1.5450	0.000	0.006	0.994	3
IPF	2.2060	0.000	0.000	1.000	3
IPF	1.3214	0.000	0.684	0.316	2
IPF	1.3132	0.000	0.721	0.279	2
IPF	1.0129	0.000	0.997	0.003	2
IPF	1.0491	0.000	0.995	0.005	2
IPF	1.4637	0.000	0.061	0.939	3
IPF	0.5581	0.998	0.002	0.000	1
IPF	0.9792	0.000	0.998	0.002	2
IPF	0.9894	0.000	0.998	0.002	2
IPF	1.0467	0.000	0.995	0.005	2
IPF	2.0241	0.000	0.000	1.000	3
IPF	1.0578	0.000	0.995	0.005	2
IPF	1.4725	0.000	0.048	0.952	3
IPF	0.9557	0.000	0.998	0.002	2
IPF	1.2121	0.000	0.950	0.050	2
IPF	1.1685	0.000	0.976	0.024	2
IPF	1.1085	0.000	0.990	0.010	2
IPF	1.1416	0.000	0.984	0.016	2
IPF	1.2312	0.000	0.931	0.069	2
IPF	1.1632	0.000	0.978	0.022	2
IPF	1.4978	0.000	0.024	0.976	3
IPF	1.6640	0.000	0.000	1.000	3
IPF	0.6410	0.978	0.022	0.000	1
IPF	1.0767	0.000	0.993	0.007	2
IPF	1.1404	0.000	0.984	0.016	2
IPF	1.5634	0.000	0.003	0.997	3
IPF	1.5628	0.000	0.004	0.996	3
IPF	1.9961	0.000	0.000	1.000	3
IPF	1.1181	0.000	0.989	0.011	2
IPF	1.0472	0.000	0.995	0.005	2
IPF	1.5478	0.000	0.006	0.994	3
IPF	0.9940	0.000	0.997	0.003	2
IPF	0.9601	0.000	0.998	0.002	2
IPF	1.1311	0.000	0.986	0.014	2
IPF	1.1434	0.000	0.984	0.016	2
IPF	1.0228	0.000	0.997	0.003	2
IPF	0.9838	0.000	0.998	0.002	2
IPF	1.0479	0.000	0.995	0.005	2
IPF	1.4858	0.000	0.034	0.966	3
IPF	0.9478	0.000	0.998	0.002	2
IPF	1.1501	0.000	0.982	0.018	2
IPF	1.7194	0.000	0.000	1.000	3
IPF	1.0355	0.000	0.996	0.004	2
IPF	1.0132	0.000	0.997	0.003	2

Trait	*FCGR3B:CD36* Raw Ratio	P1	P2	P3	Copy Number
IPF	1.6713	0.000	0.000	1.000	3
IPF	1.0989	0.000	0.991	0.009	2
IPF	0.9766	0.000	0.998	0.002	2
IPF	0.6159	0.990	0.010	0.000	1
IPF	1.1049	0.000	0.991	0.009	2
IPF	0.5693	0.998	0.002	0.000	1
IPF	1.4920	0.000	0.028	0.972	3
IPF	1.1201	0.000	0.988	0.012	2
IPF	1.5012	0.000	0.022	0.978	3
IPF	1.0456	0.000	0.996	0.004	2
IPF	1.2888	0.000	0.813	0.187	2
IPF	1.1924	0.000	0.964	0.036	2
IPF	1.1254	0.000	0.987	0.013	2
IPF	1.2433	0.000	0.915	0.085	2
IPF	1.4252	0.000	0.152	0.848	3
IPF	1.4739	0.000	0.046	0.954	3
IPF	1.6892	0.000	0.000	1.000	3
IPF	0.9269	0.000	0.998	0.001	2
IPF	1.6696	0.000	0.000	1.000	3
IPF	1.3881	0.000	0.314	0.686	3
IPF	0.9839	0.000	0.998	0.002	2
IPF	1.0226	0.000	0.997	0.003	2
IPF	1.4307	0.000	0.135	0.865	3
IPF	0.9503	0.000	0.998	0.002	2
IPF	1.1615	0.000	0.978	0.022	2
IPF	1.0718	0.000	0.994	0.006	2
IPF	1.2479	0.000	0.908	0.092	2
IPF	2.2557	0.000	0.000	1.000	3
IPF	1.1806	0.000	0.971	0.029	2
IPF	0.9777	0.000	0.998	0.002	2
IPF	0.9538	0.000	0.998	0.002	2
IPF	1.2072	0.000	0.954	0.046	2
IPF	1.2011	0.000	0.959	0.041	2
IPF	0.9289	0.000	0.998	0.002	2
IPF	1.2107	0.000	0.952	0.048	2
IPF	0.9603	0.000	0.998	0.002	2
IPF	1.6115	0.000	0.001	0.999	3
IPF	1.5406	0.000	0.007	0.993	3
IPF	0.5789	0.997	0.003	0.000	1
IPF	1.0380	0.000	0.996	0.004	2
IPF	1.1796	0.000	0.971	0.029	2
IPF	0.9389	0.000	0.998	0.002	2
IPF	1.5209	0.000	0.012	0.988	3
IPF	1.1170	0.000	0.989	0.011	2
IPF	0.9712	0.000	0.998	0.002	2
IPF	2.0041	0.000	0.000	1.000	3
IPF	1.7512	0.000	0.000	1.000	3
IPF	1.0100	0.000	0.997	0.003	2
IPF	1.1218	0.000	0.988	0.012	2

Trait	*FCGR3B:CD36* Raw Ratio	P1	P2	P3	Copy Number
IPF	1.9305	0.000	0.000	1.000	3
IPF	1.8685	0.000	0.000	1.000	3
IPF	1.1660	0.000	0.977	0.023	2
IPF	1.0130	0.000	0.997	0.003	2
IPF	1.1913	0.000	0.965	0.035	2
IPF	1.0190	0.000	0.997	0.003	2
IPF	0.9373	0.000	0.998	0.002	2
IPF	0.9853	0.000	0.998	0.002	2
IPF	0.9768	0.000	0.998	0.002	2
IPF	1.2139	0.000	0.949	0.051	2
IPF	1.0266	0.000	0.996	0.004	2
IPF	0.8827	0.000	0.998	0.001	2
IPF	0.8623	0.001	0.997	0.001	2
IPF	1.6398	0.000	0.000	1.000	3
IPF	1.0795	0.000	0.993	0.007	2
IPF	0.8379	0.006	0.993	0.001	2
IPF	1.0144	0.000	0.997	0.003	2
IPF	1.0486	0.000	0.995	0.005	2
IPF	1.0802	0.000	0.993	0.007	2
IPF	1.0940	0.000	0.992	0.008	2
IPF	1.6231	0.000	0.001	0.999	3
IPF	0.5368	0.999	0.001	0.000	1
IPF	0.9170	0.000	0.999	0.001	2
IPF	1.5121	0.000	0.016	0.984	3
IPF	1.6658	0.000	0.000	1.000	3
IPF	1.1283	0.000	0.987	0.013	2
IPF	1.0327	0.000	0.996	0.004	2
IPF	1.1235	0.000	0.988	0.012	2
IPF	1.3897	0.000	0.306	0.694	3
IPF	0.5941	0.995	0.005	0.000	1
IPF	1.6574	0.000	0.000	1.000	3
IPF	1.4870	0.000	0.033	0.967	3
IPF	0.9118	0.000	0.999	0.001	2
IPF	1.1953	0.000	0.963	0.037	2
IPF	1.0183	0.000	0.997	0.003	2
IPF	1.5652	0.000	0.003	0.997	3
IPF	1.3776	0.000	0.372	0.628	3
IPF	1.9446	0.000	0.000	1.000	3
IPF	1.0464	0.000	0.995	0.005	2
IPF	1.6262	0.000	0.000	1.000	3
IPF	1.0214	0.000	0.997	0.003	2
IPF	1.1909	0.000	0.965	0.035	2
IPF	1.1341	0.000	0.986	0.014	2
IPF	1.4634	0.000	0.061	0.939	3
IPF	0.9401	0.000	0.998	0.002	2
IPF	1.1008	0.000	0.991	0.009	2

Abbreviations: P1: probability for 1 copy; P2: probability for 2 copies; P3: probability for 3 copies; *FCGR3B*:*CD36* ratio: ratio of *FCGR3B*- to *CD36*-specific qPCR amplification.

15.2.2. LDF-transformed Data

A. Control

Trait	*FCGR3B*:*CD36* Raw Ratio	P1	P2	P3	Copy Number
Control	2.8819	0.000	0.995	0.005	2
Control	3.3171	0.000	0.978	0.022	2
Control	3.1176	0.000	0.990	0.010	2
Control	3.4758	0.000	0.954	0.046	2
Control	3.5491	0.000	0.932	0.068	2
Control	3.3410	0.000	0.976	0.024	2
Control	3.5268	0.000	0.940	0.060	2
Control	1.3823	1.000	0.000	0.000	1
Control	3.1562	0.000	0.989	0.011	2
Control	3.1697	0.000	0.988	0.012	2
Control	2.8405	0.000	0.996	0.004	2
Control	4.1972	0.000	0.134	0.866	3
Control	3.6806	0.000	0.865	0.135	2
Control	4.5709	0.000	0.005	0.995	3
Control	3.3154	0.000	0.978	0.022	2
Control	3.6261	0.000	0.899	0.101	2
Control	3.2359	0.000	0.985	0.015	2
Control	3.0658	0.000	0.992	0.008	2
Control	4.9142	0.000	0.000	1.000	3
Control	3.2917	0.000	0.980	0.020	2
Control	4.2843	0.000	0.069	0.931	3
Control	3.4588	0.000	0.957	0.043	2
Control	6.1952	0.000	0.000	1.000	3
Control	3.1608	0.000	0.989	0.011	2
Control	3.6685	0.000	0.874	0.126	2
Control	3.4199	0.000	0.965	0.035	2
Control	4.7914	0.000	0.001	0.999	3
Control	1.5584	0.999	0.001	0.000	1
Control	4.5137	0.000	0.009	0.991	3
Control	3.2694	0.000	0.982	0.018	2
Control	3.7029	0.000	0.848	0.152	2
Control	1.8406	0.970	0.030	0.000	1
Control	2.6542	0.000	0.997	0.003	2
Control	5.3990	0.000	0.000	1.000	3
Control	3.1215	0.000	0.990	0.010	2
Control	4.8320	0.000	0.000	1.000	3
Control	3.2279	0.000	0.985	0.015	2
Control	1.6548	0.997	0.003	0.000	1
Control	2.3212	0.008	0.990	0.002	2
Control	2.9793	0.000	0.994	0.006	2
Control	2.9772	0.000	0.994	0.006	2
Control	2.4799	0.000	0.997	0.002	2
Control	3.1630	0.000	0.988	0.012	2
Control	2.9948	0.000	0.994	0.006	2

Trait	*FCGR3B*:*CD36* Raw Ratio	P1	P2	P3	Copy Number
Control	2.9006	0.000	0.995	0.005	2
Control	3.1348	0.000	0.990	0.010	2
Control	3.2375	0.000	0.984	0.016	2
Control	3.1169	0.000	0.990	0.010	2
Control	4.0856	0.000	0.276	0.724	3
Control	2.9785	0.000	0.994	0.006	2
Control	2.8191	0.000	0.996	0.004	2
Control	3.4657	0.000	0.956	0.044	2
Control	6.0584	0.000	0.000	1.000	3
Control	4.3177	0.000	0.052	0.948	3
Control	3.2108	0.000	0.986	0.014	2
Control	3.0608	0.000	0.992	0.008	2
Control	4.5880	0.000	0.004	0.996	3
Control	2.6818	0.000	0.997	0.003	2
Control	3.1313	0.000	0.990	0.010	2
Control	3.1991	0.000	0.987	0.013	2
Control	4.2197	0.000	0.113	0.887	3
Control	3.3340	0.000	0.976	0.024	2
Control	1.7871	0.986	0.014	0.000	1
Control	4.5054	0.000	0.010	0.990	3
Control	3.7912	0.000	0.761	0.239	2
Control	3.0752	0.000	0.992	0.008	2
Control	3.2024	0.000	0.987	0.013	2
Control	2.2914	0.015	0.983	0.002	2
Control	4.7898	0.000	0.001	0.999	3
Control	1.8728	0.953	0.047	0.000	1
Control	2.6245	0.000	0.997	0.003	2
Control	2.8675	0.000	0.996	0.004	2
Control	3.1766	0.000	0.988	0.012	2
Control	3.0547	0.000	0.992	0.008	2
Control	2.8652	0.000	0.996	0.004	2
Control	1.3330	1.000	0.000	0.000	1
Control	2.7692	0.000	0.996	0.004	2
Control	1.7624	0.990	0.010	0.000	1
Control	4.4470	0.000	0.017	0.983	3
Control	1.1812	1.000	0.000	0.000	1
Control	3.4498	0.000	0.959	0.041	2
Control	2.3725	0.003	0.995	0.002	2
Control	3.1328	0.000	0.990	0.010	2
Control	2.8506	0.000	0.996	0.004	2
Control	2.9091	0.000	0.995	0.005	2
Control	2.6719	0.000	0.997	0.003	2
Control	2.9984	0.000	0.994	0.006	2
Control	3.1635	0.000	0.988	0.012	2
Control	3.0775	0.000	0.992	0.008	2
Control	2.8518	0.000	0.996	0.004	2
Control	1.3610	1.000	0.000	0.000	1
Control	3.3187	0.000	0.978	0.022	2
Control	2.7820	0.000	0.996	0.004	2

Trait	*FCGR3B*:*CD36* Raw Ratio	P1	P2	P3	Copy Number
Control	1.4331	1.000	0.000	0.000	1
Control	5.2834	0.000	0.000	1.000	3
Control	4.8119	0.000	0.000	1.000	3
Control	3.2728	0.000	0.982	0.018	2
Control	3.3065	0.000	0.979	0.021	2
Control	2.8296	0.000	0.996	0.004	2
Control	3.2776	0.000	0.982	0.018	2
Control	3.2496	0.000	0.984	0.016	2
Control	3.4852	0.000	0.951	0.049	2
Control	1.4491	1.000	0.000	0.000	1
Control	2.8124	0.000	0.996	0.004	2
Control	3.2703	0.000	0.982	0.018	2
Control	0.3406	1.000	0.000	0.000	1
Control	3.0431	0.000	0.992	0.008	2
Control	2.9829	0.000	0.994	0.006	2
Control	1.4488	1.000	0.000	0.000	1
Control	3.2322	0.000	0.985	0.015	2
Control	3.3083	0.000	0.979	0.021	2
Control	1.6333	0.998	0.002	0.000	1
Control	3.2546	0.000	0.983	0.017	2
Control	1.3021	1.000	0.000	0.000	1
Control	3.0663	0.000	0.992	0.008	2
Control	4.5965	0.000	0.004	0.996	3
Control	2.6422	0.000	0.997	0.003	2
Control	4.7513	0.000	0.001	0.999	3
Control	4.9841	0.000	0.000	1.000	3
Control	3.4236	0.000	0.964	0.036	2
Control	3.2972	0.000	0.980	0.020	2
Control	6.3894	0.000	0.000	1.000	3
Control	3.2230	0.000	0.985	0.015	2
Control	4.4820	0.000	0.012	0.988	3
Control	1.7046	0.995	0.005	0.000	1
Control	3.3932	0.000	0.969	0.031	2
Control	3.0832	0.000	0.991	0.009	2
Control	4.8846	0.000	0.000	1.000	3
Control	3.4638	0.000	0.956	0.044	2
Control	3.4889	0.000	0.950	0.050	2
Control	4.5470	0.000	0.006	0.994	3
Control	3.0247	0.000	0.993	0.007	2
Control	3.6061	0.000	0.909	0.091	2
Control	3.3465	0.000	0.975	0.025	2
Control	3.5441	0.000	0.934	0.066	2
Control	6.5487	0.000	0.000	1.000	3
Control	3.4699	0.000	0.955	0.045	2
Control	2.3554	0.004	0.994	0.002	2
Control	3.6859	0.000	0.861	0.139	2
Control	2.5806	0.000	0.997	0.002	2
Control	5.0634	0.000	0.000	1.000	3
Control	2.8308	0.000	0.996	0.004	2

Trait	*FCGR3B*:*CD36* Raw Ratio	P1	P2	P3	Copy Number
Control	3.4885	0.000	0.950	0.050	2
Control	3.5253	0.000	0.940	0.060	2
Control	3.3045	0.000	0.979	0.021	2
Control	4.8548	0.000	0.000	1.000	3
Control	2.8601	0.000	0.996	0.004	2
Control	3.3342	0.000	0.976	0.024	2
Control	3.5676	0.000	0.926	0.074	2
Control	3.6434	0.000	0.889	0.111	2
Control	2.1026	0.332	0.666	0.001	2
Control	1.9616	0.838	0.162	0.000	1
Control	3.5057	0.000	0.946	0.054	2
Control	3.3638	0.000	0.973	0.027	2
Control	3.1499	0.000	0.989	0.011	2
Control	3.3721	0.000	0.972	0.028	2
Control	2.6285	0.000	0.997	0.003	2
Control	3.4095	0.000	0.966	0.034	2
Control	3.0294	0.000	0.993	0.007	2
Control	3.3918	0.000	0.969	0.031	2
Control	3.1784	0.000	0.988	0.012	2
Control	4.9993	0.000	0.000	1.000	3
Control	3.4419	0.000	0.961	0.039	2
Control	2.8991	0.000	0.995	0.005	2
Control	2.5535	0.000	0.998	0.002	2
Control	3.2330	0.000	0.985	0.015	2
Control	3.1381	0.000	0.989	0.011	2
Control	1.4451	1.000	0.000	0.000	1
Control	2.9735	0.000	0.994	0.006	2
Control	3.3078	0.000	0.979	0.021	2
Control	4.5235	0.000	0.008	0.992	3
Control	2.0498	0.552	0.447	0.001	1
Control	2.9429	0.000	0.995	0.005	2
Control	3.0355	0.000	0.993	0.007	2
Control	2.8376	0.000	0.996	0.004	2
Control	3.0951	0.000	0.991	0.009	2
Control	4.9184	0.000	0.000	1.000	3
Control	3.2587	0.000	0.983	0.017	2
Control	4.4415	0.000	0.017	0.983	3
Control	3.0913	0.000	0.991	0.009	2
Control	3.2469	0.000	0.984	0.016	2
Control	2.9977	0.000	0.994	0.006	2
Control	2.3277	0.007	0.991	0.002	2
Control	3.4593	0.000	0.957	0.043	2
Control	1.7831	0.986	0.014	0.000	1
Control	1.7189	0.994	0.006	0.000	1
Control	3.5200	0.000	0.942	0.058	2
Control	3.9491	0.000	0.516	0.484	2
Control	3.3239	0.000	0.977	0.023	2
Control	3.2109	0.000	0.986	0.014	2
Control	3.5364	0.000	0.937	0.063	2

Trait	*FCGR3B*:*CD36* Raw Ratio	P1	P2	P3	Copy Number
Control	3.0680	0.000	0.992	0.008	2
Control	3.5868	0.000	0.918	0.082	2
Control	2.9781	0.000	0.994	0.006	2
Control	3.0108	0.000	0.993	0.007	2
Control	4.7284	0.000	0.001	0.999	3
Control	4.9556	0.000	0.000	1.000	3
Control	4.6183	0.000	0.003	0.997	3
Control	1.9112	0.919	0.081	0.000	1
Control	4.1759	0.000	0.155	0.845	3
Control	3.3474	0.000	0.975	0.025	2
Control	3.2237	0.000	0.985	0.015	2
Control	3.4223	0.000	0.964	0.036	2
Control	4.0665	0.000	0.306	0.694	3
Control	3.5292	0.000	0.939	0.061	2
Control	4.4604	0.000	0.015	0.985	3
Control	4.1319	0.000	0.209	0.791	3
Control	3.4763	0.000	0.953	0.047	2
Control	3.3588	0.000	0.974	0.026	2
Control	3.9471	0.000	0.520	0.480	2
Control	2.4723	0.000	0.997	0.002	2
Control	3.1815	0.000	0.988	0.012	2
Control	3.4186	0.000	0.965	0.035	2
Control	3.5498	0.000	0.932	0.068	2
Control	1.2570	1.000	0.000	0.000	1
Control	3.3646	0.000	0.973	0.027	2
Control	4.1239	0.000	0.219	0.781	3
Control	3.3507	0.000	0.974	0.026	2
Control	2.7789	0.000	0.996	0.004	2
Control	3.0037	0.000	0.993	0.007	2
Control	5.8014	0.000	0.000	1.000	3

Abbreviations: P1: probability for 1 copy; P2: probability for 2 copies; P3: probability for 3 copies; *FCGR3B*:*CD36* ratio: ratio of *FCGR3B*- to *CD36*-specific qPCR amplification.

B. IPF

Trait	*FCGR3B*:*CD36* Raw Ratio	P1	P2	P3	Copy Number
IPF	6.4669	0.000	0.000	1.000	3
IPF	4.1606	0.000	0.270	0.730	3
IPF	4.6020	0.000	0.006	0.994	3
IPF	6.5709	0.000	0.000	1.000	3
IPF	3.9362	0.000	0.684	0.316	2
IPF	3.9117	0.000	0.721	0.279	2
IPF	3.0170	0.000	0.997	0.003	2
IPF	3.1250	0.000	0.995	0.005	2
IPF	4.3598	0.000	0.061	0.939	3
IPF	1.6623	0.998	0.002	0.000	1
IPF	2.9167	0.000	0.998	0.002	2
IPF	2.9471	0.000	0.998	0.002	2
IPF	3.1177	0.000	0.995	0.005	2
IPF	6.0290	0.000	0.000	1.000	3
IPF	3.1509	0.000	0.995	0.005	2
IPF	4.3861	0.000	0.048	0.952	3
IPF	2.8466	0.000	0.998	0.002	2
IPF	3.6106	0.000	0.950	0.050	2
IPF	3.4807	0.000	0.976	0.024	2
IPF	3.3018	0.000	0.990	0.010	2
IPF	3.4005	0.000	0.984	0.016	2
IPF	3.6675	0.000	0.931	0.069	2
IPF	3.4649	0.000	0.978	0.022	2
IPF	4.4616	0.000	0.024	0.976	3
IPF	4.9566	0.000	0.000	1.000	3
IPF	1.9092	0.978	0.022	0.000	1
IPF	3.2071	0.000	0.993	0.007	2
IPF	3.3970	0.000	0.984	0.016	2
IPF	4.6570	0.000	0.003	0.997	3
IPF	4.6550	0.000	0.004	0.996	3
IPF	5.9458	0.000	0.000	1.000	3
IPF	3.3303	0.000	0.989	0.011	2
IPF	3.1194	0.000	0.995	0.005	2
IPF	4.6104	0.000	0.006	0.994	3
IPF	2.9609	0.000	0.997	0.003	2
IPF	2.8598	0.000	0.998	0.002	2
IPF	3.3692	0.000	0.986	0.014	2
IPF	3.4058	0.000	0.984	0.016	2
IPF	3.0466	0.000	0.997	0.003	2
IPF	2.9305	0.000	0.998	0.002	2
IPF	3.1212	0.000	0.995	0.005	2
IPF	4.4256	0.000	0.034	0.966	3
IPF	2.8232	0.000	0.998	0.002	2
IPF	3.4258	0.000	0.982	0.018	2
IPF	5.1216	0.000	0.000	1.000	3
IPF	3.0843	0.000	0.996	0.004	2
IPF	3.0180	0.000	0.997	0.003	2

Trait	*FCGR3B*:*CD36* Raw Ratio	P1	P2	P3	Copy Number
IPF	4.9781	0.000	0.000	1.000	3
IPF	3.2731	0.000	0.991	0.009	2
IPF	2.9090	0.000	0.998	0.002	2
IPF	1.8345	0.990	0.010	0.000	1
IPF	3.2913	0.000	0.991	0.009	2
IPF	1.6957	0.998	0.002	0.000	1
IPF	4.4441	0.000	0.028	0.972	3
IPF	3.3364	0.000	0.988	0.012	2
IPF	4.4716	0.000	0.022	0.978	3
IPF	3.1144	0.000	0.996	0.004	2
IPF	3.8389	0.000	0.813	0.187	2
IPF	3.5518	0.000	0.964	0.036	2
IPF	3.3522	0.000	0.987	0.013	2
IPF	3.7035	0.000	0.915	0.085	2
IPF	4.2452	0.000	0.152	0.848	3
IPF	4.3904	0.000	0.046	0.954	3
IPF	5.0315	0.000	0.000	1.000	3
IPF	2.7608	0.000	0.998	0.001	2
IPF	4.9731	0.000	0.000	1.000	3
IPF	4.1347	0.000	0.314	0.686	3
IPF	2.9308	0.000	0.998	0.002	2
IPF	3.0460	0.000	0.997	0.003	2
IPF	4.2616	0.000	0.135	0.865	3
IPF	2.8307	0.000	0.998	0.002	2
IPF	3.4596	0.000	0.978	0.022	2
IPF	3.1925	0.000	0.994	0.006	2
IPF	3.7171	0.000	0.908	0.092	2
IPF	6.7189	0.000	0.000	1.000	3
IPF	3.5167	0.000	0.971	0.029	2
IPF	2.9122	0.000	0.998	0.002	2
IPF	2.8412	0.000	0.998	0.002	2
IPF	3.5959	0.000	0.954	0.046	2
IPF	3.5776	0.000	0.959	0.041	2
IPF	2.7668	0.000	0.998	0.002	2
IPF	3.6064	0.000	0.952	0.048	2
IPF	2.8605	0.000	0.998	0.002	2
IPF	4.8001	0.000	0.001	0.999	3
IPF	4.5891	0.000	0.007	0.993	3
IPF	1.7245	0.997	0.003	0.000	1
IPF	3.0920	0.000	0.996	0.004	2
IPF	3.5138	0.000	0.971	0.029	2
IPF	2.7966	0.000	0.998	0.002	2
IPF	4.5303	0.000	0.012	0.988	3
IPF	3.3272	0.000	0.989	0.011	2
IPF	2.8928	0.000	0.998	0.002	2
IPF	5.9696	0.000	0.000	1.000	3
IPF	5.2164	0.000	0.000	1.000	3
IPF	3.0085	0.000	0.997	0.003	2
IPF	3.3416	0.000	0.988	0.012	2

Trait	*FCGR3B*:*CD36* Raw Ratio	P1	P2	P3	Copy Number
IPF	5.7503	0.000	0.000	1.000	3
IPF	5.5657	0.000	0.000	1.000	3
IPF	3.4731	0.000	0.977	0.023	2
IPF	3.0174	0.000	0.997	0.003	2
IPF	3.5484	0.000	0.965	0.035	2
IPF	3.0353	0.000	0.997	0.003	2
IPF	2.7918	0.000	0.998	0.002	2
IPF	2.9348	0.000	0.998	0.002	2
IPF	2.9096	0.000	0.998	0.002	2
IPF	3.6159	0.000	0.949	0.051	2
IPF	3.0578	0.000	0.996	0.004	2
IPF	2.6293	0.000	0.998	0.001	2
IPF	2.5685	0.001	0.997	0.001	2
IPF	4.8846	0.000	0.000	1.000	3
IPF	3.2156	0.000	0.993	0.007	2
IPF	2.4958	0.006	0.993	0.001	2
IPF	3.0217	0.000	0.997	0.003	2
IPF	3.1234	0.000	0.995	0.005	2
IPF	3.2177	0.000	0.993	0.007	2
IPF	3.2587	0.000	0.992	0.008	2
IPF	4.8348	0.000	0.001	0.999	3
IPF	1.5991	0.999	0.001	0.000	1
IPF	2.7314	0.000	0.999	0.001	2
IPF	4.5042	0.000	0.016	0.984	3
IPF	4.9619	0.000	0.000	1.000	3
IPF	3.3610	0.000	0.987	0.013	2
IPF	3.0760	0.000	0.996	0.004	2
IPF	3.3466	0.000	0.988	0.012	2
IPF	4.1394	0.000	0.306	0.694	3
IPF	1.7697	0.995	0.005	0.000	1
IPF	4.9370	0.000	0.000	1.000	3
IPF	4.4292	0.000	0.033	0.967	3
IPF	2.7159	0.000	0.999	0.001	2
IPF	3.5605	0.000	0.963	0.037	2
IPF	3.0331	0.000	0.997	0.003	2
IPF	4.6621	0.000	0.003	0.997	3
IPF	4.1033	0.000	0.372	0.628	3
IPF	5.7924	0.000	0.000	1.000	3
IPF	3.1168	0.000	0.995	0.005	2
IPF	4.8439	0.000	0.000	1.000	3
IPF	3.0424	0.000	0.997	0.003	2
IPF	3.5472	0.000	0.965	0.035	2
IPF	3.3780	0.000	0.986	0.014	2
IPF	4.3590	0.000	0.061	0.939	3
IPF	2.8002	0.000	0.998	0.002	2
IPF	3.2791	0.000	0.991	0.009	2

Abbreviations: P1: probability for 1 copy; P2: probability for 2 copies; P3: probability for 3 copies; *FCGR3B*:*CD36* ratio: ratio of *FCGR3B*- to *CD36*-specific qPCR amplification.

15.3 ECCS/ERS Predicted Lung Function Values

15.3.1.Males

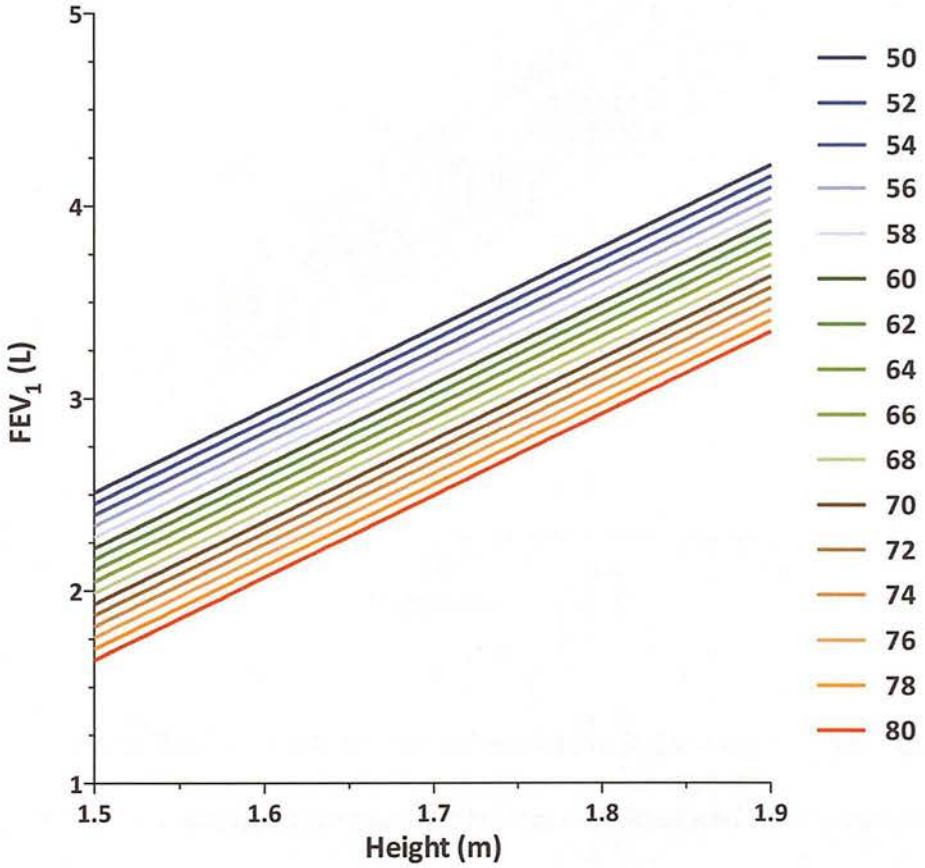

Figure 15.1.: Predicted FEV_1 (L - forced expiratory volume in 1 sec) values for males aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines.

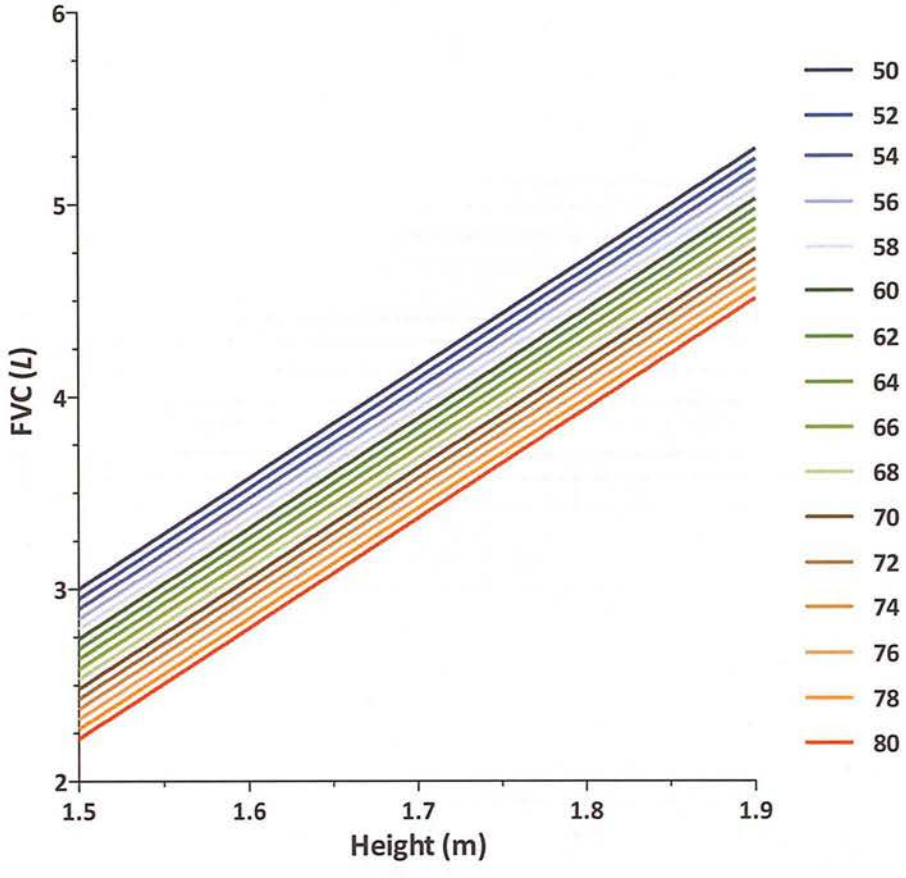

Figure 15.2.: Predicted FVC (L - forced vital capacity) values for males aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines.

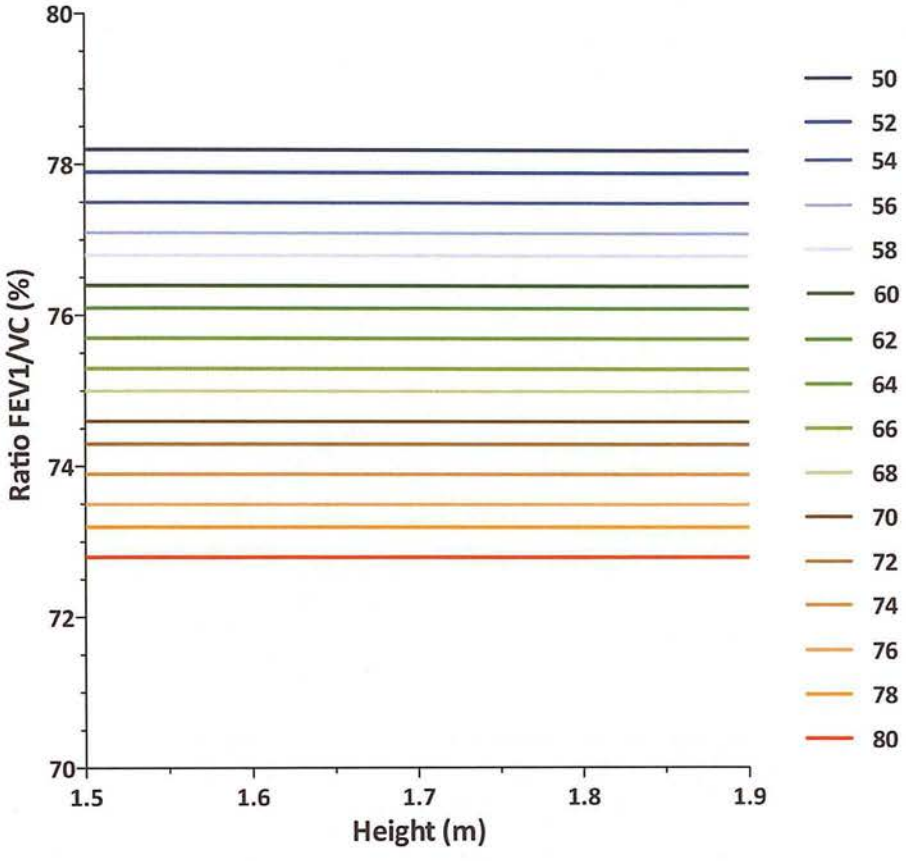

Figure 15.3.: Predicted FEV_1/VC Ratio (%) values for males aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines.

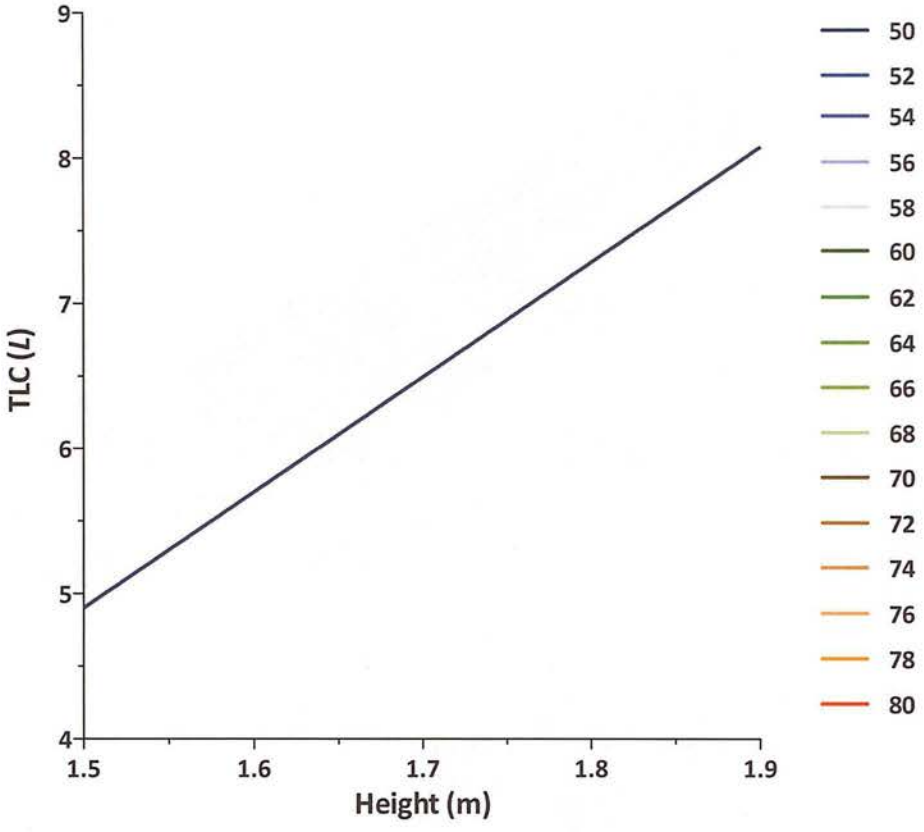

Figure 15.4.: Predicted TLC (L - total lung capacity) values for males aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines. Note that TLC values are identical irrespective of age.

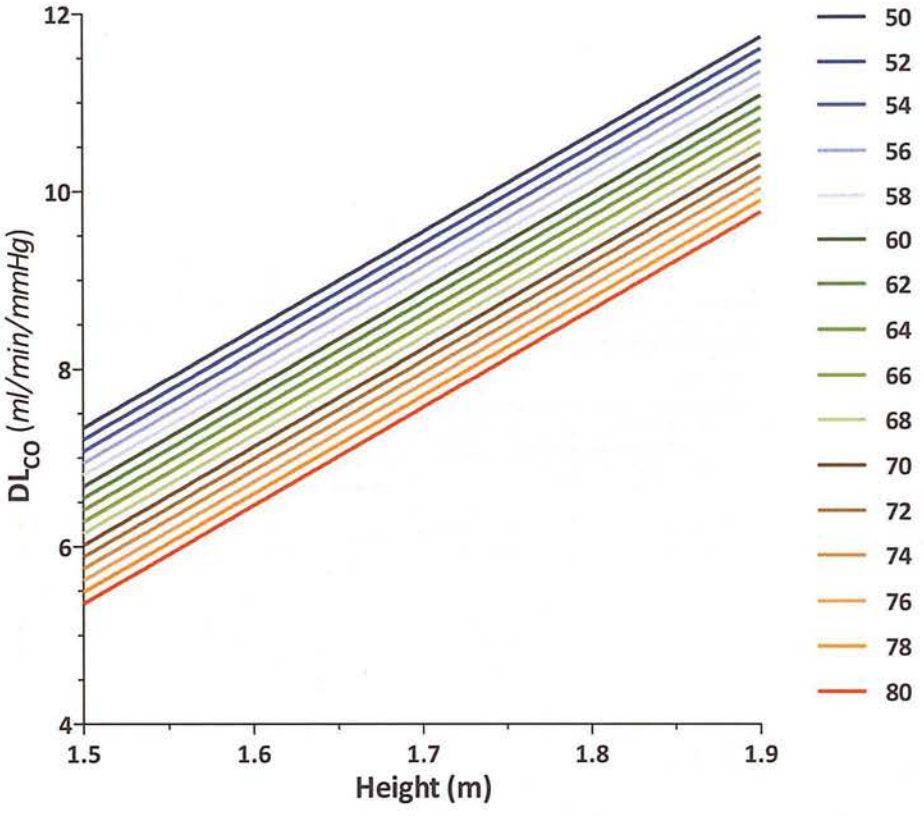

Figure 15.5.: Predicted DL_{CO} (ml $min^{-1}mmHg^{-1}$ - diffusing capacity for carbon monoxide) values for males aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines.

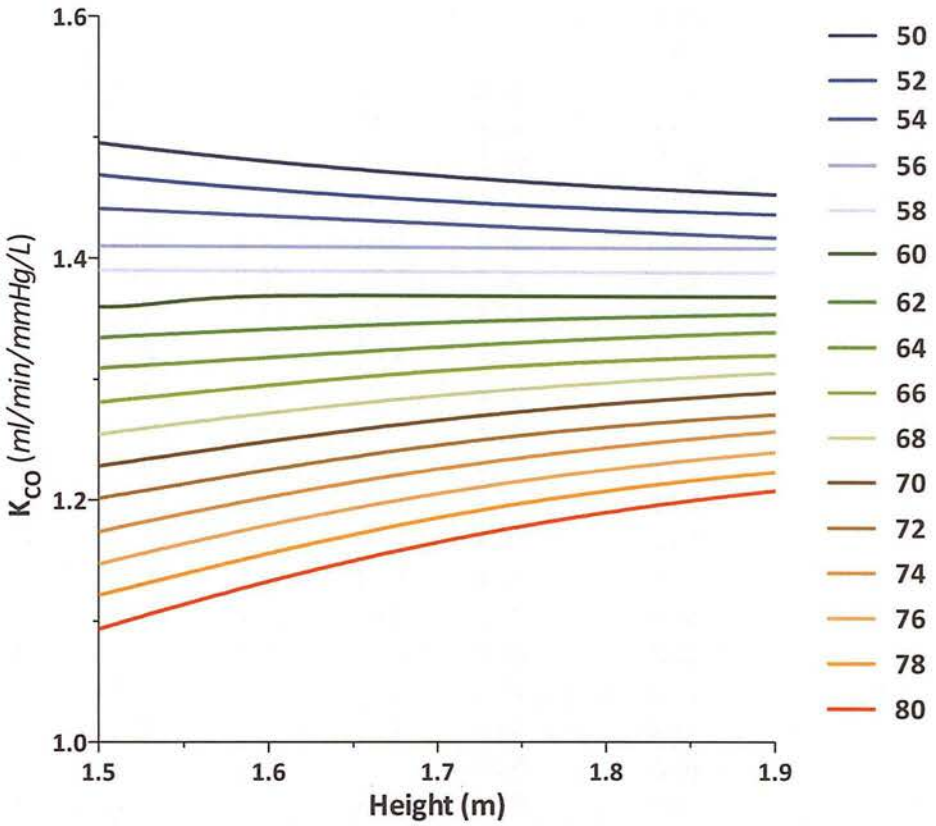

Figure 15.6.: Predicted K_{CO} (ml $min^{-1}mmHg^{-1}L^{-1}$ - DL_{CO} corrected for alveolar volume) values for males aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines.

Age= 50

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.51	3.00	78.2	4.91	7.34	1.50
1.51	2.55	3.06	78.2	4.98	7.45	1.49
1.52	2.60	3.12	78.2	5.06	7.56	1.49
1.53	2.64	3.17	78.2	5.14	7.67	1.49
1.54	2.68	3.23	78.2	5.22	7.78	1.49
1.55	2.73	3.29	78.2	5.30	7.89	1.49
1.56	2.77	3.35	78.2	5.38	8.00	1.49
1.57	2.81	3.40	78.2	5.46	8.11	1.48
1.58	2.85	3.46	78.2	5.54	8.22	1.48
1.59	2.90	3.52	78.2	5.62	8.33	1.48
1.60	2.94	3.58	78.2	5.70	8.45	1.48
1.61	2.98	3.63	78.2	5.78	8.56	1.48
1.62	3.03	3.69	78.2	5.86	8.67	1.48
1.63	3.07	3.75	78.2	5.94	8.78	1.48
1.64	3.11	3.81	78.2	6.02	8.89	1.48
1.65	3.16	3.86	78.2	6.10	9.00	1.47
1.66	3.20	3.92	78.2	6.18	9.11	1.47
1.67	3.24	3.98	78.2	6.26	9.22	1.47
1.68	3.28	4.04	78.2	6.34	9.33	1.47
1.69	3.33	4.09	78.2	6.42	9.45	1.47
1.70	3.37	4.15	78.2	6.50	9.56	1.47
1.71	3.41	4.21	78.2	6.58	9.67	1.47
1.72	3.46	4.27	78.2	6.66	9.78	1.47
1.73	3.50	4.32	78.2	6.74	9.89	1.47
1.74	3.54	4.38	78.2	6.82	10.00	1.47
1.75	3.59	4.44	78.2	6.90	10.11	1.47
1.76	3.63	4.50	78.2	6.98	10.22	1.46
1.77	3.67	4.56	78.2	7.06	10.33	1.46
1.78	3.71	4.61	78.2	7.14	10.45	1.46
1.79	3.76	4.67	78.2	7.22	10.56	1.46
1.80	3.80	4.73	78.2	7.30	10.67	1.46
1.81	3.84	4.79	78.2	7.38	10.78	1.46
1.82	3.89	4.84	78.2	7.46	10.89	1.46
1.83	3.93	4.90	78.2	7.54	11.00	1.46
1.84	3.97	4.96	78.2	7.62	11.11	1.46
1.85	4.02	5.02	78.2	7.70	11.22	1.46
1.86	4.06	5.07	78.2	7.78	11.33	1.46
1.87	4.10	5.13	78.2	7.86	11.45	1.46
1.88	4.14	5.19	78.2	7.94	11.56	1.46
1.89	4.19	5.25	78.2	8.02	11.67	1.45
1.90	4.23	5.30	78.2	8.10	11.78	1.45

Age= 52

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.45	2.95	77.9	4.91	7.20	1.47
1.51	2.50	3.01	77.9	4.98	7.31	1.47
1.52	2.54	3.06	77.9	5.06	7.43	1.47
1.53	2.58	3.12	77.9	5.14	7.54	1.46
1.54	2.62	3.18	77.9	5.22	7.65	1.46
1.55	2.67	3.24	77.9	5.30	7.76	1.46
1.56	2.71	3.29	77.9	5.38	7.87	1.46
1.57	2.75	3.35	77.9	5.46	7.98	1.46
1.58	2.80	3.41	77.9	5.54	8.09	1.46
1.59	2.84	3.47	77.9	5.62	8.20	1.46
1.60	2.88	3.52	77.9	5.70	8.31	1.46
1.61	2.93	3.58	77.9	5.78	8.43	1.46
1.62	2.97	3.64	77.9	5.86	8.54	1.46
1.63	3.01	3.70	77.9	5.94	8.65	1.45
1.64	3.05	3.75	77.9	6.02	8.76	1.45
1.65	3.10	3.81	77.9	6.10	8.87	1.45
1.66	3.14	3.87	77.9	6.18	8.98	1.45
1.67	3.18	3.93	77.9	6.26	9.09	1.45
1.68	3.23	3.98	77.9	6.34	9.20	1.45
1.69	3.27	4.04	77.9	6.42	9.31	1.45
1.70	3.31	4.10	77.9	6.50	9.43	1.45
1.71	3.36	4.16	77.9	6.58	9.54	1.45
1.72	3.40	4.22	77.9	6.66	9.65	1.45
1.73	3.44	4.27	77.9	6.74	9.76	1.45
1.74	3.48	4.33	77.9	6.82	9.87	1.45
1.75	3.53	4.39	77.9	6.90	9.98	1.45
1.76	3.57	4.45	77.9	6.98	10.09	1.45
1.77	3.61	4.50	77.9	7.06	10.20	1.44
1.78	3.66	4.56	77.9	7.14	10.31	1.44
1.79	3.70	4.62	77.9	7.22	10.42	1.44
1.80	3.74	4.68	77.9	7.30	10.54	1.44
1.81	3.79	4.73	77.9	7.38	10.65	1.44
1.82	3.83	4.79	77.9	7.46	10.76	1.44
1.83	3.87	4.85	77.9	7.54	10.87	1.44
1.84	3.91	4.91	77.9	7.62	10.98	1.44
1.85	3.96	4.96	77.9	7.70	11.09	1.44
1.86	4.00	5.02	77.9	7.78	11.20	1.44
1.87	4.04	5.08	77.9	7.86	11.31	1.44
1.88	4.09	5.14	77.9	7.94	11.42	1.44
1.89	4.13	5.19	77.9	8.02	11.54	1.44
1.90	4.17	5.25	77.9	8.10	11.65	1.44

Age= 54

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.39	2.90	77.5	4.91	7.07	1.44
1.51	2.44	2.95	77.5	4.98	7.18	1.44
1.52	2.48	3.01	77.5	5.06	7.29	1.44
1.53	2.52	3.07	77.5	5.14	7.40	1.44
1.54	2.57	3.13	77.5	5.22	7.52	1.44
1.55	2.61	3.18	77.5	5.30	7.63	1.44
1.56	2.65	3.24	77.5	5.38	7.74	1.44
1.57	2.70	3.30	77.5	5.46	7.85	1.44
1.58	2.74	3.36	77.5	5.54	7.96	1.44
1.59	2.78	3.41	77.5	5.62	8.07	1.44
1.60	2.82	3.47	77.5	5.70	8.18	1.43
1.61	2.87	3.53	77.5	5.78	8.29	1.43
1.62	2.91	3.59	77.5	5.86	8.40	1.43
1.63	2.95	3.64	77.5	5.94	8.52	1.43
1.64	3.00	3.70	77.5	6.02	8.63	1.43
1.65	3.04	3.76	77.5	6.10	8.74	1.43
1.66	3.08	3.82	77.5	6.18	8.85	1.43
1.67	3.13	3.88	77.5	6.26	8.96	1.43
1.68	3.17	3.93	77.5	6.34	9.07	1.43
1.69	3.21	3.99	77.5	6.42	9.18	1.43
1.70	3.25	4.05	77.5	6.50	9.29	1.43
1.71	3.30	4.11	77.5	6.58	9.40	1.43
1.72	3.34	4.16	77.5	6.66	9.52	1.43
1.73	3.38	4.22	77.5	6.74	9.63	1.43
1.74	3.43	4.28	77.5	6.82	9.74	1.43
1.75	3.47	4.34	77.5	6.90	9.85	1.43
1.76	3.51	4.39	77.5	6.98	9.96	1.43
1.77	3.56	4.45	77.5	7.06	10.07	1.43
1.78	3.60	4.51	77.5	7.14	10.18	1.43
1.79	3.64	4.57	77.5	7.22	10.29	1.43
1.80	3.68	4.62	77.5	7.30	10.40	1.42
1.81	3.73	4.68	77.5	7.38	10.52	1.42
1.82	3.77	4.74	77.5	7.46	10.63	1.42
1.83	3.81	4.80	77.5	7.54	10.74	1.42
1.84	3.86	4.85	77.5	7.62	10.85	1.42
1.85	3.90	4.91	77.5	7.70	10.96	1.42
1.86	3.94	4.97	77.5	7.78	11.07	1.42
1.87	3.99	5.03	77.5	7.86	11.18	1.42
1.88	4.03	5.08	77.5	7.94	11.29	1.42
1.89	4.07	5.14	77.5	8.02	11.40	1.42
1.90	4.11	5.20	77.5	8.10	11.52	1.42

Age=56

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.34	2.84	77.1	4.91	6.94	1.41
1.51	2.38	2.90	77.1	4.98	7.05	1.41
1.52	2.42	2.96	77.1	5.06	7.16	1.41
1.53	2.47	3.02	77.1	5.14	7.27	1.41
1.54	2.51	3.07	77.1	5.22	7.38	1.41
1.55	2.55	3.13	77.1	5.30	7.49	1.41
1.56	2.59	3.19	77.1	5.38	7.61	1.41
1.57	2.64	3.25	77.1	5.46	7.72	1.41
1.58	2.68	3.30	77.1	5.54	7.83	1.41
1.59	2.72	3.36	77.1	5.62	7.94	1.41
1.60	2.77	3.42	77.1	5.70	8.05	1.41
1.61	2.81	3.48	77.1	5.78	8.16	1.41
1.62	2.85	3.54	77.1	5.86	8.27	1.41
1.63	2.90	3.59	77.1	5.94	8.38	1.41
1.64	2.94	3.65	77.1	6.02	8.49	1.41
1.65	2.98	3.71	77.1	6.10	8.61	1.41
1.66	3.02	3.77	77.1	6.18	8.72	1.41
1.67	3.07	3.82	77.1	6.26	8.83	1.41
1.68	3.11	3.88	77.1	6.34	8.94	1.41
1.69	3.15	3.94	77.1	6.42	9.05	1.41
1.70	3.20	4.00	77.1	6.50	9.16	1.41
1.71	3.24	4.05	77.1	6.58	9.27	1.41
1.72	3.28	4.11	77.1	6.66	9.38	1.41
1.73	3.33	4.17	77.1	6.74	9.49	1.41
1.74	3.37	4.23	77.1	6.82	9.61	1.41
1.75	3.41	4.28	77.1	6.90	9.72	1.41
1.76	3.45	4.34	77.1	6.98	9.83	1.41
1.77	3.50	4.40	77.1	7.06	9.94	1.41
1.78	3.54	4.46	77.1	7.14	10.05	1.41
1.79	3.58	4.51	77.1	7.22	10.16	1.41
1.80	3.63	4.57	77.1	7.30	10.27	1.41
1.81	3.67	4.63	77.1	7.38	10.38	1.41
1.82	3.71	4.69	77.1	7.46	10.49	1.41
1.83	3.76	4.74	77.1	7.54	10.61	1.41
1.84	3.80	4.80	77.1	7.62	10.72	1.41
1.85	3.84	4.86	77.1	7.70	10.83	1.41
1.86	3.88	4.92	77.1	7.78	10.94	1.41
1.87	3.93	4.98	77.1	7.86	11.05	1.41
1.88	3.97	5.03	77.1	7.94	11.16	1.41
1.89	4.01	5.09	77.1	8.02	11.27	1.41
1.90	4.06	5.15	77.1	8.10	11.38	1.41

Age=58

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.28	2.79	76.8	4.91	6.81	1.39
1.51	2.32	2.85	76.8	4.98	6.92	1.39
1.52	2.36	2.91	76.8	5.06	7.03	1.39
1.53	2.41	2.96	76.8	5.14	7.14	1.39
1.54	2.45	3.02	76.8	5.22	7.25	1.39
1.55	2.49	3.08	76.8	5.30	7.36	1.39
1.56	2.54	3.14	76.8	5.38	7.47	1.39
1.57	2.58	3.20	76.8	5.46	7.58	1.39
1.58	2.62	3.25	76.8	5.54	7.70	1.39
1.59	2.67	3.31	76.8	5.62	7.81	1.39
1.60	2.71	3.37	76.8	5.70	7.92	1.39
1.61	2.75	3.43	76.8	5.78	8.03	1.39
1.62	2.79	3.48	76.8	5.86	8.14	1.39
1.63	2.84	3.54	76.8	5.94	8.25	1.39
1.64	2.88	3.60	76.8	6.02	8.36	1.39
1.65	2.92	3.66	76.8	6.10	8.47	1.39
1.66	2.97	3.71	76.8	6.18	8.58	1.39
1.67	3.01	3.77	76.8	6.26	8.70	1.39
1.68	3.05	3.83	76.8	6.34	8.81	1.39
1.69	3.10	3.89	76.8	6.42	8.92	1.39
1.70	3.14	3.94	76.8	6.50	9.03	1.39
1.71	3.18	4.00	76.8	6.58	9.14	1.39
1.72	3.22	4.06	76.8	6.66	9.25	1.39
1.73	3.27	4.12	76.8	6.74	9.36	1.39
1.74	3.31	4.17	76.8	6.82	9.47	1.39
1.75	3.35	4.23	76.8	6.90	9.58	1.39
1.76	3.40	4.29	76.8	6.98	9.70	1.39
1.77	3.44	4.35	76.8	7.06	9.81	1.39
1.78	3.48	4.40	76.8	7.14	9.92	1.39
1.79	3.53	4.46	76.8	7.22	10.03	1.39
1.80	3.57	4.52	76.8	7.30	10.14	1.39
1.81	3.61	4.58	76.8	7.38	10.25	1.39
1.82	3.65	4.64	76.8	7.46	10.36	1.39
1.83	3.70	4.69	76.8	7.54	10.47	1.39
1.84	3.74	4.75	76.8	7.62	10.58	1.39
1.85	3.78	4.81	76.8	7.70	10.70	1.39
1.86	3.83	4.87	76.8	7.78	10.81	1.39
1.87	3.87	4.92	76.8	7.86	10.92	1.39
1.88	3.91	4.98	76.8	7.94	11.03	1.39
1.89	3.96	5.04	76.8	8.02	11.14	1.39
1.90	4.00	5.10	76.8	8.10	11.25	1.39

Age= 60

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.22	2.74	76.4	4.91	6.68	1.36
1.51	2.26	2.80	76.4	4.98	6.79	1.36
1.52	2.31	2.86	76.4	5.06	6.90	1.36
1.53	2.35	2.91	76.4	5.14	7.01	1.36
1.54	2.39	2.97	76.4	5.22	7.12	1.36
1.55	2.44	3.03	76.4	5.30	7.23	1.36
1.56	2.48	3.09	76.4	5.38	7.34	1.36
1.57	2.52	3.14	76.4	5.46	7.45	1.36
1.58	2.56	3.20	76.4	5.54	7.56	1.36
1.59	2.61	3.26	76.4	5.62	7.67	1.36
1.60	2.65	3.32	76.4	5.70	7.79	1.37
1.61	2.69	3.37	76.4	5.78	7.90	1.37
1.62	2.74	3.43	76.4	5.86	8.01	1.37
1.63	2.78	3.49	76.4	5.94	8.12	1.37
1.64	2.82	3.55	76.4	6.02	8.23	1.37
1.65	2.87	3.60	76.4	6.10	8.34	1.37
1.66	2.91	3.66	76.4	6.18	8.45	1.37
1.67	2.95	3.72	76.4	6.26	8.56	1.37
1.68	2.99	3.78	76.4	6.34	8.67	1.37
1.69	3.04	3.83	76.4	6.42	8.79	1.37
1.70	3.08	3.89	76.4	6.50	8.90	1.37
1.71	3.12	3.95	76.4	6.58	9.01	1.37
1.72	3.17	4.01	76.4	6.66	9.12	1.37
1.73	3.21	4.06	76.4	6.74	9.23	1.37
1.74	3.25	4.12	76.4	6.82	9.34	1.37
1.75	3.30	4.18	76.4	6.90	9.45	1.37
1.76	3.34	4.24	76.4	6.98	9.56	1.37
1.77	3.38	4.30	76.4	7.06	9.67	1.37
1.78	3.42	4.35	76.4	7.14	9.79	1.37
1.79	3.47	4.41	76.4	7.22	9.90	1.37
1.80	3.51	4.47	76.4	7.30	10.01	1.37
1.81	3.55	4.53	76.4	7.38	10.12	1.37
1.82	3.60	4.58	76.4	7.46	10.23	1.37
1.83	3.64	4.64	76.4	7.54	10.34	1.37
1.84	3.68	4.70	76.4	7.62	10.45	1.37
1.85	3.73	4.76	76.4	7.70	10.56	1.37
1.86	3.77	4.81	76.4	7.78	10.67	1.37
1.87	3.81	4.87	76.4	7.86	10.79	1.37
1.88	3.85	4.93	76.4	7.94	10.90	1.37
1.89	3.90	4.99	76.4	8.02	11.01	1.37
1.90	3.94	5.04	76.4	8.10	11.12	1.37

Age= 62

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.16	2.69	76.1	4.91	6.54	1.33
1.51	2.21	2.75	76.1	4.98	6.65	1.33
1.52	2.25	2.80	76.1	5.06	6.77	1.34
1.53	2.29	2.86	76.1	5.14	6.88	1.34
1.54	2.33	2.92	76.1	5.22	6.99	1.34
1.55	2.38	2.98	76.1	5.30	7.10	1.34
1.56	2.42	3.03	76.1	5.38	7.21	1.34
1.57	2.46	3.09	76.1	5.46	7.32	1.34
1.58	2.51	3.15	76.1	5.54	7.43	1.34
1.59	2.55	3.21	76.1	5.62	7.54	1.34
1.60	2.59	3.26	76.1	5.70	7.65	1.34
1.61	2.64	3.32	76.1	5.78	7.77	1.34
1.62	2.68	3.38	76.1	5.86	7.88	1.34
1.63	2.72	3.44	76.1	5.94	7.99	1.34
1.64	2.76	3.49	76.1	6.02	8.10	1.34
1.65	2.81	3.55	76.1	6.10	8.21	1.35
1.66	2.85	3.61	76.1	6.18	8.32	1.35
1.67	2.89	3.67	76.1	6.26	8.43	1.35
1.68	2.94	3.72	76.1	6.34	8.54	1.35
1.69	2.98	3.78	76.1	6.42	8.65	1.35
1.70	3.02	3.84	76.1	6.50	8.77	1.35
1.71	3.07	3.90	76.1	6.58	8.88	1.35
1.72	3.11	3.96	76.1	6.66	8.99	1.35
1.73	3.15	4.01	76.1	6.74	9.10	1.35
1.74	3.19	4.07	76.1	6.82	9.21	1.35
1.75	3.24	4.13	76.1	6.90	9.32	1.35
1.76	3.28	4.19	76.1	6.98	9.43	1.35
1.77	3.32	4.24	76.1	7.06	9.54	1.35
1.78	3.37	4.30	76.1	7.14	9.65	1.35
1.79	3.41	4.36	76.1	7.22	9.76	1.35
1.80	3.45	4.42	76.1	7.30	9.88	1.35
1.81	3.50	4.47	76.1	7.38	9.99	1.35
1.82	3.54	4.53	76.1	7.46	10.10	1.35
1.83	3.58	4.59	76.1	7.54	10.21	1.35
1.84	3.62	4.65	76.1	7.62	10.32	1.35
1.85	3.67	4.70	76.1	7.70	10.43	1.35
1.86	3.71	4.76	76.1	7.78	10.54	1.35
1.87	3.75	4.82	76.1	7.86	10.65	1.36
1.88	3.80	4.88	76.1	7.94	10.76	1.36
1.89	3.84	4.93	76.1	8.02	10.88	1.36
1.90	3.88	4.99	76.1	8.10	10.99	1.36

Age= 64

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.10	2.64	75.7	4.91	6.41	1.31
1.51	2.15	2.69	75.7	4.98	6.52	1.31
1.52	2.19	2.75	75.7	5.06	6.63	1.31
1.53	2.23	2.81	75.7	5.14	6.74	1.31
1.54	2.28	2.87	75.7	5.22	6.86	1.31
1.55	2.32	2.92	75.7	5.30	6.97	1.31
1.56	2.36	2.98	75.7	5.38	7.08	1.31
1.57	2.41	3.04	75.7	5.46	7.19	1.32
1.58	2.45	3.10	75.7	5.54	7.30	1.32
1.59	2.49	3.15	75.7	5.62	7.41	1.32
1.60	2.53	3.21	75.7	5.70	7.52	1.32
1.61	2.58	3.27	75.7	5.78	7.63	1.32
1.62	2.62	3.33	75.7	5.86	7.74	1.32
1.63	2.66	3.38	75.7	5.94	7.86	1.32
1.64	2.71	3.44	75.7	6.02	7.97	1.32
1.65	2.75	3.50	75.7	6.10	8.08	1.32
1.66	2.79	3.56	75.7	6.18	8.19	1.32
1.67	2.84	3.62	75.7	6.26	8.30	1.33
1.68	2.88	3.67	75.7	6.34	8.41	1.33
1.69	2.92	3.73	75.7	6.42	8.52	1.33
1.70	2.96	3.79	75.7	6.50	8.63	1.33
1.71	3.01	3.85	75.7	6.58	8.74	1.33
1.72	3.05	3.90	75.7	6.66	8.86	1.33
1.73	3.09	3.96	75.7	6.74	8.97	1.33
1.74	3.14	4.02	75.7	6.82	9.08	1.33
1.75	3.18	4.08	75.7	6.90	9.19	1.33
1.76	3.22	4.13	75.7	6.98	9.30	1.33
1.77	3.27	4.19	75.7	7.06	9.41	1.33
1.78	3.31	4.25	75.7	7.14	9.52	1.33
1.79	3.35	4.31	75.7	7.22	9.63	1.33
1.80	3.39	4.36	75.7	7.30	9.74	1.33
1.81	3.44	4.42	75.7	7.38	9.86	1.34
1.82	3.48	4.48	75.7	7.46	9.97	1.34
1.83	3.52	4.54	75.7	7.54	10.08	1.34
1.84	3.57	4.59	75.7	7.62	10.19	1.34
1.85	3.61	4.65	75.7	7.70	10.30	1.34
1.86	3.65	4.71	75.7	7.78	10.41	1.34
1.87	3.70	4.77	75.7	7.86	10.52	1.34
1.88	3.74	4.82	75.7	7.94	10.63	1.34
1.89	3.78	4.88	75.7	8.02	10.74	1.34
1.90	3.82	4.94	75.7	8.10	10.86	1.34

Age= 66

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.05	2.58	75.3	4.91	6.28	1.28
1.51	2.09	2.64	75.3	4.98	6.39	1.28
1.52	2.13	2.70	75.3	5.06	6.50	1.28
1.53	2.18	2.76	75.3	5.14	6.61	1.29
1.54	2.22	2.81	75.3	5.22	6.72	1.29
1.55	2.26	2.87	75.3	5.30	6.83	1.29
1.56	2.30	2.93	75.3	5.38	6.95	1.29
1.57	2.35	2.99	75.3	5.46	7.06	1.29
1.58	2.39	3.04	75.3	5.54	7.17	1.29
1.59	2.43	3.10	75.3	5.62	7.28	1.29
1.60	2.48	3.16	75.3	5.70	7.39	1.30
1.61	2.52	3.22	75.3	5.78	7.50	1.30
1.62	2.56	3.28	75.3	5.86	7.61	1.30
1.63	2.61	3.33	75.3	5.94	7.72	1.30
1.64	2.65	3.39	75.3	6.02	7.83	1.30
1.65	2.69	3.45	75.3	6.10	7.95	1.30
1.66	2.73	3.51	75.3	6.18	8.06	1.30
1.67	2.78	3.56	75.3	6.26	8.17	1.30
1.68	2.82	3.62	75.3	6.34	8.28	1.31
1.69	2.86	3.68	75.3	6.42	8.39	1.31
1.70	2.91	3.74	75.3	6.50	8.50	1.31
1.71	2.95	3.79	75.3	6.58	8.61	1.31
1.72	2.99	3.85	75.3	6.66	8.72	1.31
1.73	3.04	3.91	75.3	6.74	8.83	1.31
1.74	3.08	3.97	75.3	6.82	8.95	1.31
1.75	3.12	4.02	75.3	6.90	9.06	1.31
1.76	3.16	4.08	75.3	6.98	9.17	1.31
1.77	3.21	4.14	75.3	7.06	9.28	1.31
1.78	3.25	4.20	75.3	7.14	9.39	1.31
1.79	3.29	4.25	75.3	7.22	9.50	1.32
1.80	3.34	4.31	75.3	7.30	9.61	1.32
1.81	3.38	4.37	75.3	7.38	9.72	1.32
1.82	3.42	4.43	75.3	7.46	9.83	1.32
1.83	3.47	4.48	75.3	7.54	9.95	1.32
1.84	3.51	4.54	75.3	7.62	10.06	1.32
1.85	3.55	4.60	75.3	7.70	10.17	1.32
1.86	3.59	4.66	75.3	7.78	10.28	1.32
1.87	3.64	4.72	75.3	7.86	10.39	1.32
1.88	3.68	4.77	75.3	7.94	10.50	1.32
1.89	3.72	4.83	75.3	8.02	10.61	1.32
1.90	3.77	4.89	75.3	8.10	10.72	1.32

Age= 68

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.99	2.53	75.0	4.91	6.15	1.25
1.51	2.03	2.59	75.0	4.98	6.26	1.26
1.52	2.07	2.65	75.0	5.06	6.37	1.26
1.53	2.12	2.70	75.0	5.14	6.48	1.26
1.54	2.16	2.76	75.0	5.22	6.59	1.26
1.55	2.20	2.82	75.0	5.30	6.70	1.26
1.56	2.25	2.88	75.0	5.38	6.81	1.27
1.57	2.29	2.94	75.0	5.46	6.92	1.27
1.58	2.33	2.99	75.0	5.54	7.04	1.27
1.59	2.38	3.05	75.0	5.62	7.15	1.27
1.60	2.42	3.11	75.0	5.70	7.26	1.27
1.61	2.46	3.17	75.0	5.78	7.37	1.27
1.62	2.50	3.22	75.0	5.86	7.48	1.28
1.63	2.55	3.28	75.0	5.94	7.59	1.28
1.64	2.59	3.34	75.0	6.02	7.70	1.28
1.65	2.63	3.40	75.0	6.10	7.81	1.28
1.66	2.68	3.45	75.0	6.18	7.92	1.28
1.67	2.72	3.51	75.0	6.26	8.04	1.28
1.68	2.76	3.57	75.0	6.34	8.15	1.28
1.69	2.81	3.63	75.0	6.42	8.26	1.29
1.70	2.85	3.68	75.0	6.50	8.37	1.29
1.71	2.89	3.74	75.0	6.58	8.48	1.29
1.72	2.93	3.80	75.0	6.66	8.59	1.29
1.73	2.98	3.86	75.0	6.74	8.70	1.29
1.74	3.02	3.91	75.0	6.82	8.81	1.29
1.75	3.06	3.97	75.0	6.90	8.92	1.29
1.76	3.11	4.03	75.0	6.98	9.04	1.29
1.77	3.15	4.09	75.0	7.06	9.15	1.30
1.78	3.19	4.14	75.0	7.14	9.26	1.30
1.79	3.24	4.20	75.0	7.22	9.37	1.30
1.80	3.28	4.26	75.0	7.30	9.48	1.30
1.81	3.32	4.32	75.0	7.38	9.59	1.30
1.82	3.36	4.38	75.0	7.46	9.70	1.30
1.83	3.41	4.43	75.0	7.54	9.81	1.30
1.84	3.45	4.49	75.0	7.62	9.92	1.30
1.85	3.49	4.55	75.0	7.70	10.04	1.30
1.86	3.54	4.61	75.0	7.78	10.15	1.30
1.87	3.58	4.66	75.0	7.86	10.26	1.30
1.88	3.62	4.72	75.0	7.94	10.37	1.31
1.89	3.67	4.78	75.0	8.02	10.48	1.31
1.90	3.71	4.84	75.0	8.10	10.59	1.31

Age= 70

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.93	2.48	74.6	4.91	6.02	1.23
1.51	1.97	2.54	74.6	4.98	6.13	1.23
1.52	2.02	2.60	74.6	5.06	6.24	1.23
1.53	2.06	2.65	74.6	5.14	6.35	1.23
1.54	2.10	2.71	74.6	5.22	6.46	1.24
1.55	2.15	2.77	74.6	5.30	6.57	1.24
1.56	2.19	2.83	74.6	5.38	6.68	1.24
1.57	2.23	2.88	74.6	5.46	6.79	1.24
1.58	2.27	2.94	74.6	5.54	6.90	1.25
1.59	2.32	3.00	74.6	5.62	7.01	1.25
1.60	2.36	3.06	74.6	5.70	7.13	1.25
1.61	2.40	3.11	74.6	5.78	7.24	1.25
1.62	2.45	3.17	74.6	5.86	7.35	1.25
1.63	2.49	3.23	74.6	5.94	7.46	1.25
1.64	2.53	3.29	74.6	6.02	7.57	1.26
1.65	2.58	3.34	74.6	6.10	7.68	1.26
1.66	2.62	3.40	74.6	6.18	7.79	1.26
1.67	2.66	3.46	74.6	6.26	7.90	1.26
1.68	2.70	3.52	74.6	6.34	8.01	1.26
1.69	2.75	3.57	74.6	6.42	8.13	1.27
1.70	2.79	3.63	74.6	6.50	8.24	1.27
1.71	2.83	3.69	74.6	6.58	8.35	1.27
1.72	2.88	3.75	74.6	6.66	8.46	1.27
1.73	2.92	3.80	74.6	6.74	8.57	1.27
1.74	2.96	3.86	74.6	6.82	8.68	1.27
1.75	3.01	3.92	74.6	6.90	8.79	1.27
1.76	3.05	3.98	74.6	6.98	8.90	1.28
1.77	3.09	4.04	74.6	7.06	9.01	1.28
1.78	3.13	4.09	74.6	7.14	9.13	1.28
1.79	3.18	4.15	74.6	7.22	9.24	1.28
1.80	3.22	4.21	74.6	7.30	9.35	1.28
1.81	3.26	4.27	74.6	7.38	9.46	1.28
1.82	3.31	4.32	74.6	7.46	9.57	1.28
1.83	3.35	4.38	74.6	7.54	9.68	1.28
1.84	3.39	4.44	74.6	7.62	9.79	1.28
1.85	3.44	4.50	74.6	7.70	9.90	1.29
1.86	3.48	4.55	74.6	7.78	10.01	1.29
1.87	3.52	4.61	74.6	7.86	10.13	1.29
1.88	3.56	4.67	74.6	7.94	10.24	1.29
1.89	3.61	4.73	74.6	8.02	10.35	1.29
1.90	3.65	4.78	74.6	8.10	10.46	1.29

Age= 72

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.87	2.43	74.3	4.91	5.88	1.20
1.51	1.92	2.49	74.3	4.98	5.99	1.20
1.52	1.96	2.54	74.3	5.06	6.11	1.21
1.53	2.00	2.60	74.3	5.14	6.22	1.21
1.54	2.04	2.66	74.3	5.22	6.33	1.21
1.55	2.09	2.72	74.3	5.30	6.44	1.21
1.56	2.13	2.77	74.3	5.38	6.55	1.22
1.57	2.17	2.83	74.3	5.46	6.66	1.22
1.58	2.22	2.89	74.3	5.54	6.77	1.22
1.59	2.26	2.95	74.3	5.62	6.88	1.22
1.60	2.30	3.00	74.3	5.70	6.99	1.23
1.61	2.35	3.06	74.3	5.78	7.11	1.23
1.62	2.39	3.12	74.3	5.86	7.22	1.23
1.63	2.43	3.18	74.3	5.94	7.33	1.23
1.64	2.47	3.23	74.3	6.02	7.44	1.23
1.65	2.52	3.29	74.3	6.10	7.55	1.24
1.66	2.56	3.35	74.3	6.18	7.66	1.24
1.67	2.60	3.41	74.3	6.26	7.77	1.24
1.68	2.65	3.46	74.3	6.34	7.88	1.24
1.69	2.69	3.52	74.3	6.42	7.99	1.24
1.70	2.73	3.58	74.3	6.50	8.11	1.25
1.71	2.78	3.64	74.3	6.58	8.22	1.25
1.72	2.82	3.70	74.3	6.66	8.33	1.25
1.73	2.86	3.75	74.3	6.74	8.44	1.25
1.74	2.90	3.81	74.3	6.82	8.55	1.25
1.75	2.95	3.87	74.3	6.90	8.66	1.25
1.76	2.99	3.93	74.3	6.98	8.77	1.26
1.77	3.03	3.98	74.3	7.06	8.88	1.26
1.78	3.08	4.04	74.3	7.14	8.99	1.26
1.79	3.12	4.10	74.3	7.22	9.10	1.26
1.80	3.16	4.16	74.3	7.30	9.22	1.26
1.81	3.21	4.21	74.3	7.38	9.33	1.26
1.82	3.25	4.27	74.3	7.46	9.44	1.26
1.83	3.29	4.33	74.3	7.54	9.55	1.27
1.84	3.33	4.39	74.3	7.62	9.66	1.27
1.85	3.38	4.44	74.3	7.70	9.77	1.27
1.86	3.42	4.50	74.3	7.78	9.88	1.27
1.87	3.46	4.56	74.3	7.86	9.99	1.27
1.88	3.51	4.62	74.3	7.94	10.10	1.27
1.89	3.55	4.67	74.3	8.02	10.22	1.27
1.90	3.59	4.73	74.3	8.10	10.33	1.27

Age= 74

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.81	2.38	73.9	4.91	5.75	1.17
1.51	1.86	2.43	73.9	4.98	5.86	1.18
1.52	1.90	2.49	73.9	5.06	5.97	1.18
1.53	1.94	2.55	73.9	5.14	6.08	1.18
1.54	1.99	2.61	73.9	5.22	6.20	1.19
1.55	2.03	2.66	73.9	5.30	6.31	1.19
1.56	2.07	2.72	73.9	5.38	6.42	1.19
1.57	2.12	2.78	73.9	5.46	6.53	1.19
1.58	2.16	2.84	73.9	5.54	6.64	1.20
1.59	2.20	2.89	73.9	5.62	6.75	1.20
1.60	2.24	2.95	73.9	5.70	6.86	1.20
1.61	2.29	3.01	73.9	5.78	6.97	1.21
1.62	2.33	3.07	73.9	5.86	7.08	1.21
1.63	2.37	3.12	73.9	5.94	7.20	1.21
1.64	2.42	3.18	73.9	6.02	7.31	1.21
1.65	2.46	3.24	73.9	6.10	7.42	1.22
1.66	2.50	3.30	73.9	6.18	7.53	1.22
1.67	2.55	3.36	73.9	6.26	7.64	1.22
1.68	2.59	3.41	73.9	6.34	7.75	1.22
1.69	2.63	3.47	73.9	6.42	7.86	1.22
1.70	2.67	3.53	73.9	6.50	7.97	1.23
1.71	2.72	3.59	73.9	6.58	8.08	1.23
1.72	2.76	3.64	73.9	6.66	8.20	1.23
1.73	2.80	3.70	73.9	6.74	8.31	1.23
1.74	2.85	3.76	73.9	6.82	8.42	1.23
1.75	2.89	3.82	73.9	6.90	8.53	1.24
1.76	2.93	3.87	73.9	6.98	8.64	1.24
1.77	2.98	3.93	73.9	7.06	8.75	1.24
1.78	3.02	3.99	73.9	7.14	8.86	1.24
1.79	3.06	4.05	73.9	7.22	8.97	1.24
1.80	3.10	4.10	73.9	7.30	9.08	1.24
1.81	3.15	4.16	73.9	7.38	9.20	1.25
1.82	3.19	4.22	73.9	7.46	9.31	1.25
1.83	3.23	4.28	73.9	7.54	9.42	1.25
1.84	3.28	4.33	73.9	7.62	9.53	1.25
1.85	3.32	4.39	73.9	7.70	9.64	1.25
1.86	3.36	4.45	73.9	7.78	9.75	1.25
1.87	3.41	4.51	73.9	7.86	9.86	1.25
1.88	3.45	4.56	73.9	7.94	9.97	1.26
1.89	3.49	4.62	73.9	8.02	10.08	1.26
1.90	3.53	4.68	73.9	8.10	10.20	1.26

Age= 76

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.76	2.32	73.5	4.91	5.62	1.15
1.51	1.80	2.38	73.5	4.98	5.73	1.15
1.52	1.84	2.44	73.5	5.06	5.84	1.15
1.53	1.89	2.50	73.5	5.14	5.95	1.16
1.54	1.93	2.55	73.5	5.22	6.06	1.16
1.55	1.97	2.61	73.5	5.30	6.17	1.16
1.56	2.01	2.67	73.5	5.38	6.29	1.17
1.57	2.06	2.73	73.5	5.46	6.40	1.17
1.58	2.10	2.78	73.5	5.54	6.51	1.17
1.59	2.14	2.84	73.5	5.62	6.62	1.18
1.60	2.19	2.90	73.5	5.70	6.73	1.18
1.61	2.23	2.96	73.5	5.78	6.84	1.18
1.62	2.27	3.02	73.5	5.86	6.95	1.19
1.63	2.32	3.07	73.5	5.94	7.06	1.19
1.64	2.36	3.13	73.5	6.02	7.17	1.19
1.65	2.40	3.19	73.5	6.10	7.29	1.19
1.66	2.44	3.25	73.5	6.18	7.40	1.20
1.67	2.49	3.30	73.5	6.26	7.51	1.20
1.68	2.53	3.36	73.5	6.34	7.62	1.20
1.69	2.57	3.42	73.5	6.42	7.73	1.20
1.70	2.62	3.48	73.5	6.50	7.84	1.21
1.71	2.66	3.53	73.5	6.58	7.95	1.21
1.72	2.70	3.59	73.5	6.66	8.06	1.21
1.73	2.75	3.65	73.5	6.74	8.17	1.21
1.74	2.79	3.71	73.5	6.82	8.29	1.21
1.75	2.83	3.76	73.5	6.90	8.40	1.22
1.76	2.87	3.82	73.5	6.98	8.51	1.22
1.77	2.92	3.88	73.5	7.06	8.62	1.22
1.78	2.96	3.94	73.5	7.14	8.73	1.22
1.79	3.00	3.99	73.5	7.22	8.84	1.22
1.80	3.05	4.05	73.5	7.30	8.95	1.23
1.81	3.09	4.11	73.5	7.38	9.06	1.23
1.82	3.13	4.17	73.5	7.46	9.17	1.23
1.83	3.18	4.22	73.5	7.54	9.29	1.23
1.84	3.22	4.28	73.5	7.62	9.40	1.23
1.85	3.26	4.34	73.5	7.70	9.51	1.23
1.86	3.30	4.40	73.5	7.78	9.62	1.24
1.87	3.35	4.46	73.5	7.86	9.73	1.24
1.88	3.39	4.51	73.5	7.94	9.84	1.24
1.89	3.43	4.57	73.5	8.02	9.95	1.24
1.90	3.48	4.63	73.5	8.10	10.06	1.24

Age= 78

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.70	2.27	73.2	4.91	5.49	1.12
1.51	1.74	2.33	73.2	4.98	5.60	1.12
1.52	1.78	2.39	73.2	5.06	5.71	1.13
1.53	1.83	2.44	73.2	5.14	5.82	1.13
1.54	1.87	2.50	73.2	5.22	5.93	1.14
1.55	1.91	2.56	73.2	5.30	6.04	1.14
1.56	1.96	2.62	73.2	5.38	6.15	1.14
1.57	2.00	2.68	73.2	5.46	6.26	1.15
1.58	2.04	2.73	73.2	5.54	6.38	1.15
1.59	2.09	2.79	73.2	5.62	6.49	1.15
1.60	2.13	2.85	73.2	5.70	6.60	1.16
1.61	2.17	2.91	73.2	5.78	6.71	1.16
1.62	2.21	2.96	73.2	5.86	6.82	1.16
1.63	2.26	3.02	73.2	5.94	6.93	1.17
1.64	2.30	3.08	73.2	6.02	7.04	1.17
1.65	2.34	3.14	73.2	6.10	7.15	1.17
1.66	2.39	3.19	73.2	6.18	7.26	1.17
1.67	2.43	3.25	73.2	6.26	7.38	1.18
1.68	2.47	3.31	73.2	6.34	7.49	1.18
1.69	2.52	3.37	73.2	6.42	7.60	1.18
1.70	2.56	3.42	73.2	6.50	7.71	1.19
1.71	2.60	3.48	73.2	6.58	7.82	1.19
1.72	2.64	3.54	73.2	6.66	7.93	1.19
1.73	2.69	3.60	73.2	6.74	8.04	1.19
1.74	2.73	3.65	73.2	6.82	8.15	1.20
1.75	2.77	3.71	73.2	6.90	8.26	1.20
1.76	2.82	3.77	73.2	6.98	8.38	1.20
1.77	2.86	3.83	73.2	7.06	8.49	1.20
1.78	2.90	3.88	73.2	7.14	8.60	1.20
1.79	2.95	3.94	73.2	7.22	8.71	1.21
1.80	2.99	4.00	73.2	7.30	8.82	1.21
1.81	3.03	4.06	73.2	7.38	8.93	1.21
1.82	3.07	4.12	73.2	7.46	9.04	1.21
1.83	3.12	4.17	73.2	7.54	9.15	1.21
1.84	3.16	4.23	73.2	7.62	9.26	1.22
1.85	3.20	4.29	73.2	7.70	9.38	1.22
1.86	3.25	4.35	73.2	7.78	9.49	1.22
1.87	3.29	4.40	73.2	7.86	9.60	1.22
1.88	3.33	4.46	73.2	7.94	9.71	1.22
1.89	3.38	4.52	73.2	8.02	9.82	1.22
1.90	3.42	4.58	73.2	8.10	9.93	1.23

Age= 80

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.64	2.22	72.8	4.91	5.36	1.09
1.51	1.68	2.28	72.8	4.98	5.47	1.10
1.52	1.73	2.34	72.8	5.06	5.58	1.10
1.53	1.77	2.39	72.8	5.14	5.69	1.11
1.54	1.81	2.45	72.8	5.22	5.80	1.11
1.55	1.86	2.51	72.8	5.30	5.91	1.11
1.56	1.90	2.57	72.8	5.38	6.02	1.12
1.57	1.94	2.62	72.8	5.46	6.13	1.12
1.58	1.98	2.68	72.8	5.54	6.24	1.13
1.59	2.03	2.74	72.8	5.62	6.35	1.13
1.60	2.07	2.80	72.8	5.70	6.47	1.13
1.61	2.11	2.85	72.8	5.78	6.58	1.14
1.62	2.16	2.91	72.8	5.86	6.69	1.14
1.63	2.20	2.97	72.8	5.94	6.80	1.14
1.64	2.24	3.03	72.8	6.02	6.91	1.15
1.65	2.29	3.08	72.8	6.10	7.02	1.15
1.66	2.33	3.14	72.8	6.18	7.13	1.15
1.67	2.37	3.20	72.8	6.26	7.24	1.16
1.68	2.41	3.26	72.8	6.34	7.35	1.16
1.69	2.46	3.31	72.8	6.42	7.47	1.16
1.70	2.50	3.37	72.8	6.50	7.58	1.17
1.71	2.54	3.43	72.8	6.58	7.69	1.17
1.72	2.59	3.49	72.8	6.66	7.80	1.17
1.73	2.63	3.54	72.8	6.74	7.91	1.17
1.74	2.67	3.60	72.8	6.82	8.02	1.18
1.75	2.72	3.66	72.8	6.90	8.13	1.18
1.76	2.76	3.72	72.8	6.98	8.24	1.18
1.77	2.80	3.78	72.8	7.06	8.35	1.18
1.78	2.84	3.83	72.8	7.14	8.47	1.19
1.79	2.89	3.89	72.8	7.22	8.58	1.19
1.80	2.93	3.95	72.8	7.30	8.69	1.19
1.81	2.97	4.01	72.8	7.38	8.80	1.19
1.82	3.02	4.06	72.8	7.46	8.91	1.19
1.83	3.06	4.12	72.8	7.54	9.02	1.20
1.84	3.10	4.18	72.8	7.62	9.13	1.20
1.85	3.15	4.24	72.8	7.70	9.24	1.20
1.86	3.19	4.29	72.8	7.78	9.35	1.20
1.87	3.23	4.35	72.8	7.86	9.47	1.20
1.88	3.27	4.41	72.8	7.94	9.58	1.21
1.89	3.32	4.47	72.8	8.02	9.69	1.21
1.90	3.36	4.52	72.8	8.10	9.80	1.21

15.3.2. Females

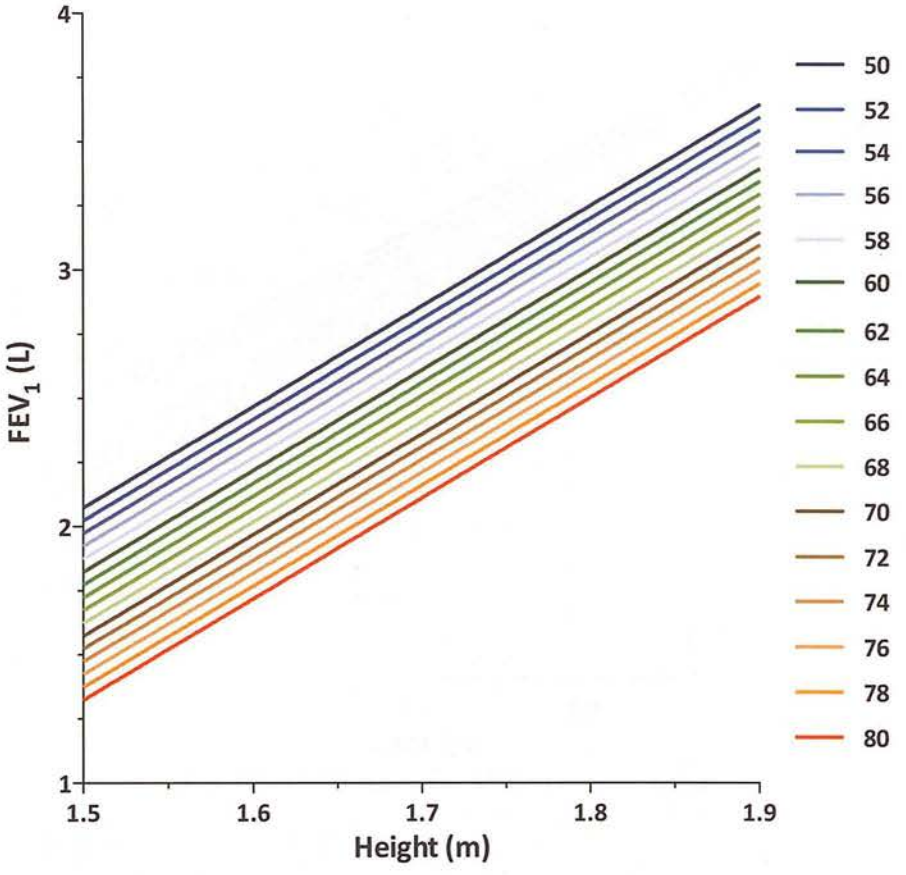

Figure 15.7.: Predicted FEV_1 (L - forced expiratory volume in 1 sec) values for females aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines.

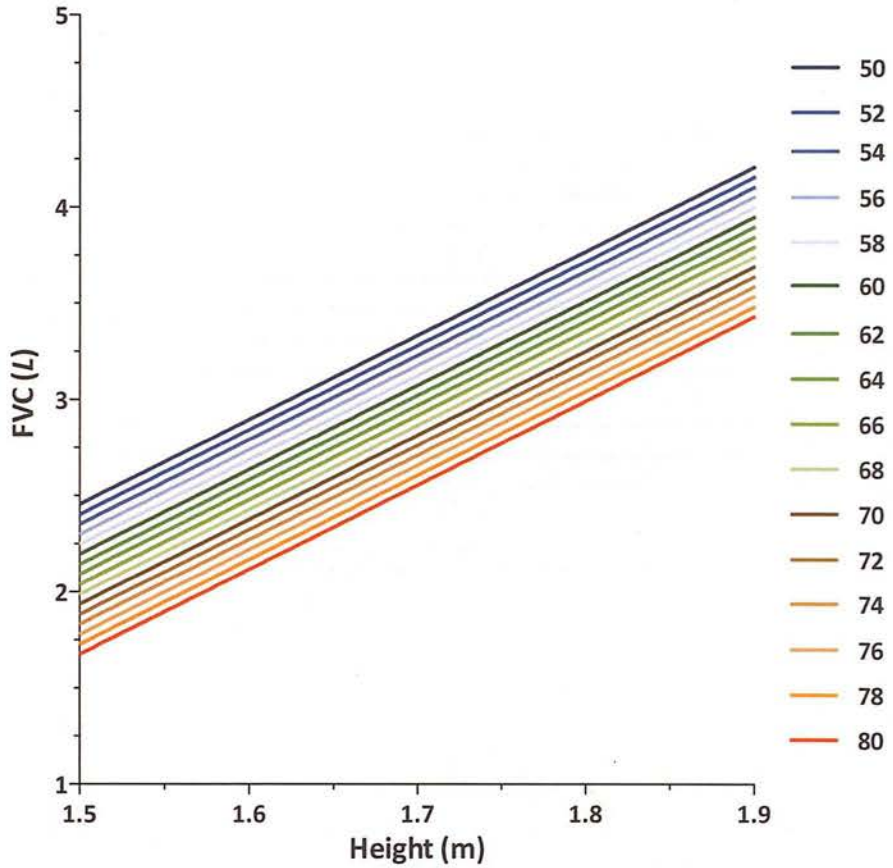

Figure 15.8.: Predicted FVC (L - forced vital capacity) values for females aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines.

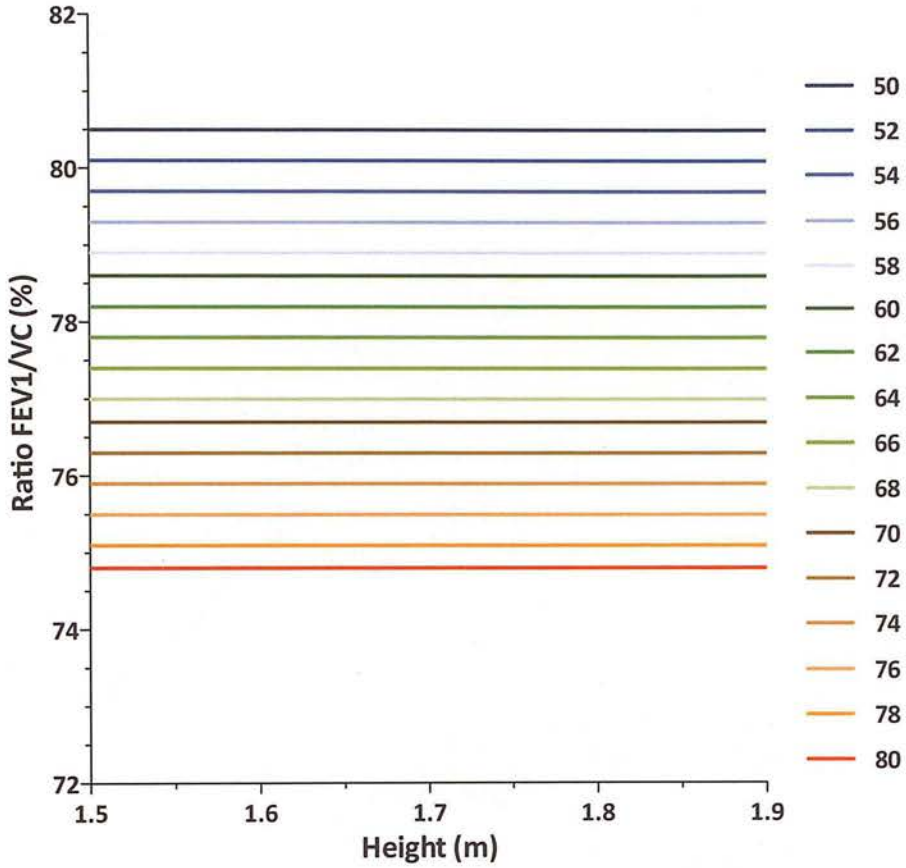

Figure 15.9.: Predicted FEV_1/VC Ratio (%) values for females aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines.

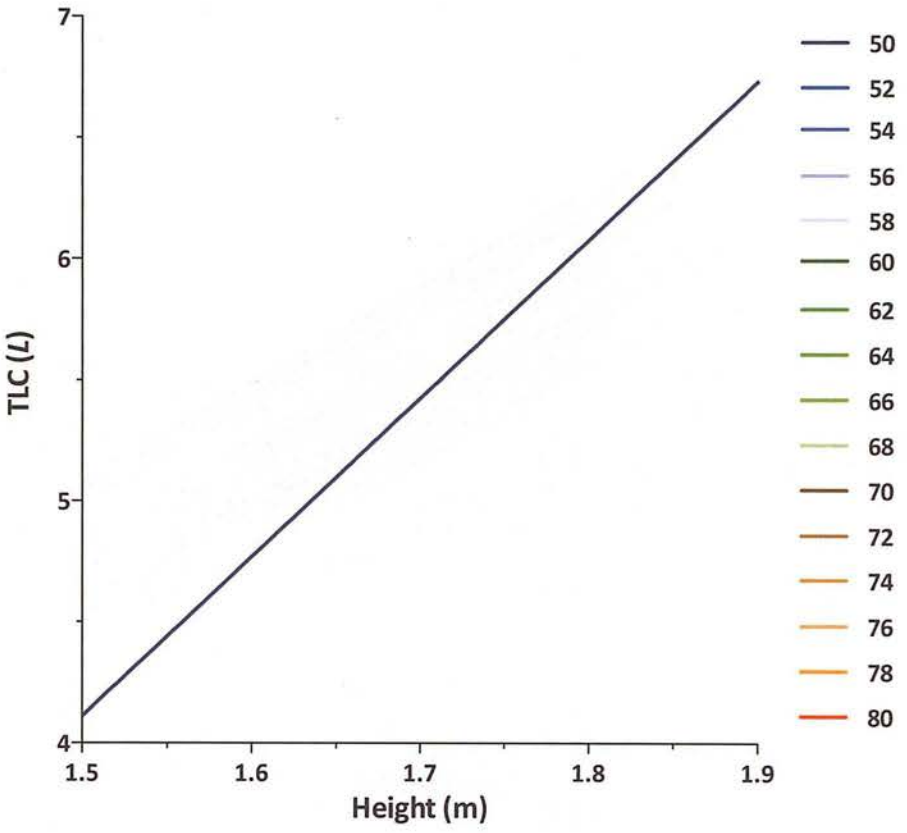

Figure 15.10.: Predicted TLC (L - total lung capacity) values for females aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines. Note that TLC values are identical irrespective of age.

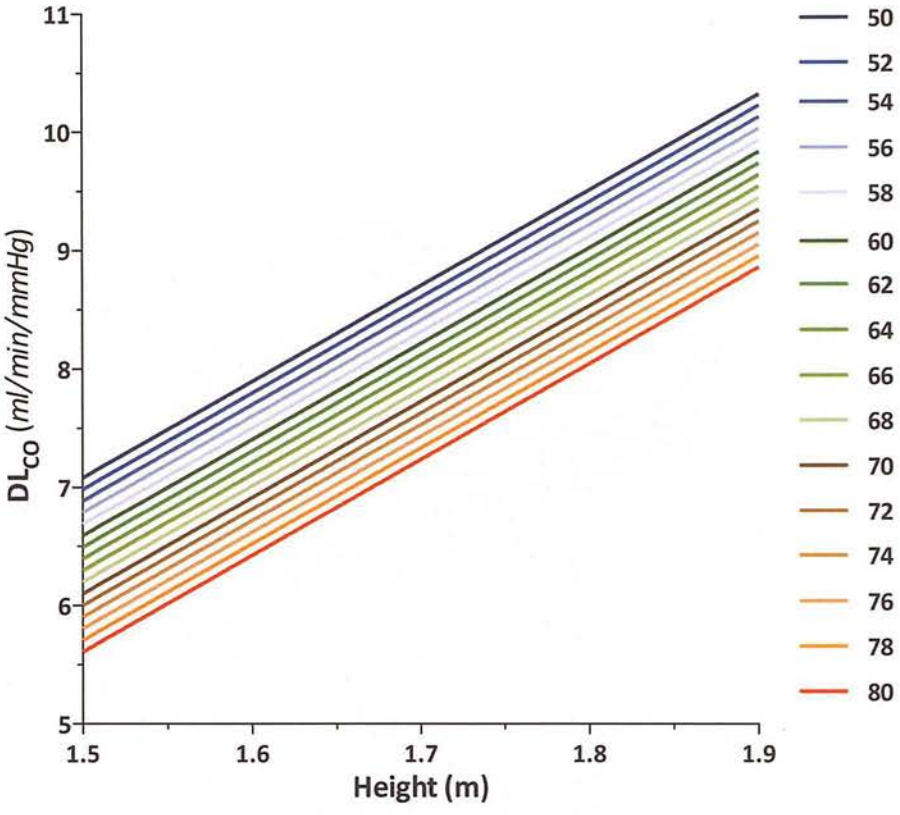

Figure 15.11.: Predicted DL_{CO} (ml $min^{-1}mmHg^{-1}$ - diffusing capacity for carbon monoxide) values for females aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines.

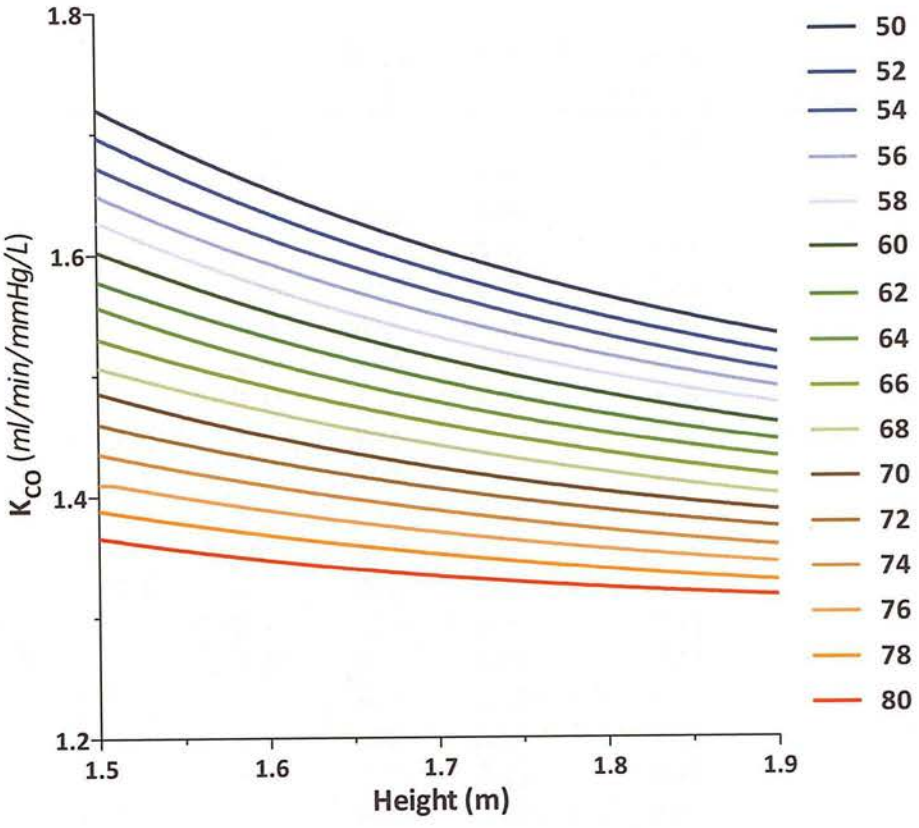

Figure 15.12.: Predicted K_{CO} (ml $min^{-1}mmHg^{-1}L^{-1}$ - DL_{CO} corrected for alveolar volume) values for females aged 50-80 based on the European Community for Coal and Steel and the European Respiratory Society guidelines.

Age= 50

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.08	2.46	80.5	4.11	7.08	1.72
1.51	2.11	2.50	80.5	4.18	7.16	1.71
1.52	2.15	2.54	80.5	4.24	7.24	1.71
1.53	2.19	2.59	80.5	4.31	7.33	1.70
1.54	2.23	2.63	80.5	4.37	7.41	1.69
1.55	2.27	2.68	80.5	4.44	7.49	1.69
1.56	2.31	2.72	80.5	4.51	7.57	1.68
1.57	2.35	2.77	80.5	4.57	7.65	1.67
1.58	2.39	2.81	80.5	4.64	7.73	1.67
1.59	2.43	2.85	80.5	4.70	7.82	1.66
1.60	2.47	2.90	80.5	4.77	7.90	1.66
1.61	2.51	2.94	80.5	4.84	7.98	1.65
1.62	2.55	2.99	80.5	4.90	8.06	1.64
1.63	2.59	3.03	80.5	4.97	8.14	1.64
1.64	2.63	3.08	80.5	5.03	8.23	1.63
1.65	2.67	3.12	80.5	5.10	8.31	1.63
1.66	2.71	3.16	80.5	5.17	8.39	1.62
1.67	2.75	3.21	80.5	5.23	8.47	1.62
1.68	2.79	3.25	80.5	5.30	8.55	1.61
1.69	2.83	3.30	80.5	5.36	8.63	1.61
1.70	2.87	3.34	80.5	5.43	8.72	1.61
1.71	2.90	3.39	80.5	5.50	8.80	1.60
1.72	2.94	3.43	80.5	5.56	8.88	1.60
1.73	2.98	3.47	80.5	5.63	8.96	1.59
1.74	3.02	3.52	80.5	5.69	9.04	1.59
1.75	3.06	3.56	80.5	5.76	9.13	1.58
1.76	3.10	3.61	80.5	5.83	9.21	1.58
1.77	3.14	3.65	80.5	5.89	9.29	1.58
1.78	3.18	3.70	80.5	5.96	9.37	1.57
1.79	3.22	3.74	80.5	6.02	9.45	1.57
1.80	3.26	3.78	80.5	6.09	9.53	1.57
1.81	3.30	3.83	80.5	6.16	9.62	1.56
1.82	3.34	3.87	80.5	6.22	9.70	1.56
1.83	3.38	3.92	80.5	6.29	9.78	1.56
1.84	3.42	3.96	80.5	6.35	9.86	1.55
1.85	3.46	4.01	80.5	6.42	9.94	1.55
1.86	3.50	4.05	80.5	6.49	10.02	1.55
1.87	3.54	4.09	80.5	6.55	10.11	1.54
1.88	3.58	4.14	80.5	6.62	10.19	1.54
1.89	3.62	4.18	80.5	6.68	10.27	1.54
1.90	3.66	4.23	80.5	6.75	10.35	1.53

Age= 52

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	2.03	2.40	80.1	4.11	6.98	1.70
1.51	2.06	2.45	80.1	4.18	7.06	1.69
1.52	2.10	2.49	80.1	4.24	7.15	1.68
1.53	2.14	2.54	80.1	4.31	7.23	1.68
1.54	2.18	2.58	80.1	4.37	7.31	1.67
1.55	2.22	2.62	80.1	4.44	7.39	1.66
1.56	2.26	2.67	80.1	4.51	7.47	1.66
1.57	2.30	2.71	80.1	4.57	7.55	1.65
1.58	2.34	2.76	80.1	4.64	7.64	1.65
1.59	2.38	2.80	80.1	4.70	7.72	1.64
1.60	2.42	2.85	80.1	4.77	7.80	1.64
1.61	2.46	2.89	80.1	4.84	7.88	1.63
1.62	2.50	2.93	80.1	4.90	7.96	1.62
1.63	2.54	2.98	80.1	4.97	8.05	1.62
1.64	2.58	3.02	80.1	5.03	8.13	1.61
1.65	2.62	3.07	80.1	5.10	8.21	1.61
1.66	2.66	3.11	80.1	5.17	8.29	1.60
1.67	2.70	3.16	80.1	5.23	8.37	1.60
1.68	2.74	3.20	80.1	5.30	8.45	1.60
1.69	2.78	3.24	80.1	5.36	8.54	1.59
1.70	2.82	3.29	80.1	5.43	8.62	1.59
1.71	2.85	3.33	80.1	5.50	8.70	1.58
1.72	2.89	3.38	80.1	5.56	8.78	1.58
1.73	2.93	3.42	80.1	5.63	8.86	1.57
1.74	2.97	3.47	80.1	5.69	8.95	1.57
1.75	3.01	3.51	80.1	5.76	9.03	1.57
1.76	3.05	3.55	80.1	5.83	9.11	1.56
1.77	3.09	3.60	80.1	5.89	9.19	1.56
1.78	3.13	3.64	80.1	5.96	9.27	1.56
1.79	3.17	3.69	80.1	6.02	9.35	1.55
1.80	3.21	3.73	80.1	6.09	9.44	1.55
1.81	3.25	3.78	80.1	6.16	9.52	1.55
1.82	3.29	3.82	80.1	6.22	9.60	1.54
1.83	3.33	3.86	80.1	6.29	9.68	1.54
1.84	3.37	3.91	80.1	6.35	9.76	1.54
1.85	3.41	3.95	80.1	6.42	9.85	1.53
1.86	3.45	4.00	80.1	6.49	9.93	1.53
1.87	3.49	4.04	80.1	6.55	10.01	1.53
1.88	3.53	4.09	80.1	6.62	10.09	1.52
1.89	3.57	4.13	80.1	6.68	10.17	1.52
1.90	3.61	4.18	80.1	6.75	10.25	1.52

Age= 54

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.98	2.35	79.7	4.11	6.88	1.67
1.51	2.01	2.40	79.7	4.18	6.97	1.67
1.52	2.05	2.44	79.7	4.24	7.05	1.66
1.53	2.09	2.48	79.7	4.31	7.13	1.65
1.54	2.13	2.53	79.7	4.37	7.21	1.65
1.55	2.17	2.57	79.7	4.44	7.29	1.64
1.56	2.21	2.62	79.7	4.51	7.37	1.64
1.57	2.25	2.66	79.7	4.57	7.46	1.63
1.58	2.29	2.71	79.7	4.64	7.54	1.63
1.59	2.33	2.75	79.7	4.70	7.62	1.62
1.60	2.37	2.79	79.7	4.77	7.70	1.61
1.61	2.41	2.84	79.7	4.84	7.78	1.61
1.62	2.45	2.88	79.7	4.90	7.87	1.60
1.63	2.49	2.93	79.7	4.97	7.95	1.60
1.64	2.53	2.97	79.7	5.03	8.03	1.59
1.65	2.57	3.02	79.7	5.10	8.11	1.59
1.66	2.61	3.06	79.7	5.17	8.19	1.59
1.67	2.65	3.10	79.7	5.23	8.27	1.58
1.68	2.69	3.15	79.7	5.30	8.36	1.58
1.69	2.73	3.19	79.7	5.36	8.44	1.57
1.70	2.77	3.24	79.7	5.43	8.52	1.57
1.71	2.80	3.28	79.7	5.50	8.60	1.57
1.72	2.84	3.33	79.7	5.56	8.68	1.56
1.73	2.88	3.37	79.7	5.63	8.77	1.56
1.74	2.92	3.41	79.7	5.69	8.85	1.55
1.75	2.96	3.46	79.7	5.76	8.93	1.55
1.76	3.00	3.50	79.7	5.83	9.01	1.55
1.77	3.04	3.55	79.7	5.89	9.09	1.54
1.78	3.08	3.59	79.7	5.96	9.17	1.54
1.79	3.12	3.64	79.7	6.02	9.26	1.54
1.80	3.16	3.68	79.7	6.09	9.34	1.53
1.81	3.20	3.72	79.7	6.16	9.42	1.53
1.82	3.24	3.77	79.7	6.22	9.50	1.53
1.83	3.28	3.81	79.7	6.29	9.58	1.52
1.84	3.32	3.86	79.7	6.35	9.67	1.52
1.85	3.36	3.90	79.7	6.42	9.75	1.52
1.86	3.40	3.95	79.7	6.49	9.83	1.52
1.87	3.44	3.99	79.7	6.55	9.91	1.51
1.88	3.48	4.03	79.7	6.62	9.99	1.51
1.89	3.52	4.08	79.7	6.68	10.07	1.51
1.90	3.56	4.12	79.7	6.75	10.16	1.50

Age= 56

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.93	2.30	79.3	4.11	6.79	1.65
1.51	1.96	2.34	79.3	4.18	6.87	1.64
1.52	2.00	2.39	79.3	4.24	6.95	1.64
1.53	2.04	2.43	79.3	4.31	7.03	1.63
1.54	2.08	2.48	79.3	4.37	7.11	1.63
1.55	2.12	2.52	79.3	4.44	7.20	1.62
1.56	2.16	2.56	79.3	4.51	7.28	1.61
1.57	2.20	2.61	79.3	4.57	7.36	1.61
1.58	2.24	2.65	79.3	4.64	7.44	1.60
1.59	2.28	2.70	79.3	4.70	7.52	1.60
1.60	2.32	2.74	79.3	4.77	7.60	1.59
1.61	2.36	2.79	79.3	4.84	7.69	1.59
1.62	2.40	2.83	79.3	4.90	7.77	1.58
1.63	2.44	2.87	79.3	4.97	7.85	1.58
1.64	2.48	2.92	79.3	5.03	7.93	1.58
1.65	2.52	2.96	79.3	5.10	8.01	1.57
1.66	2.56	3.01	79.3	5.17	8.09	1.57
1.67	2.60	3.05	79.3	5.23	8.18	1.56
1.68	2.64	3.10	79.3	5.30	8.26	1.56
1.69	2.68	3.14	79.3	5.36	8.34	1.55
1.70	2.72	3.19	79.3	5.43	8.42	1.55
1.71	2.75	3.23	79.3	5.50	8.50	1.55
1.72	2.79	3.27	79.3	5.56	8.59	1.54
1.73	2.83	3.32	79.3	5.63	8.67	1.54
1.74	2.87	3.36	79.3	5.69	8.75	1.54
1.75	2.91	3.41	79.3	5.76	8.83	1.53
1.76	2.95	3.45	79.3	5.83	8.91	1.53
1.77	2.99	3.50	79.3	5.89	8.99	1.53
1.78	3.03	3.54	79.3	5.96	9.08	1.52
1.79	3.07	3.58	79.3	6.02	9.16	1.52
1.80	3.11	3.63	79.3	6.09	9.24	1.52
1.81	3.15	3.67	79.3	6.16	9.32	1.51
1.82	3.19	3.72	79.3	6.22	9.40	1.51
1.83	3.23	3.76	79.3	6.29	9.49	1.51
1.84	3.27	3.81	79.3	6.35	9.57	1.51
1.85	3.31	3.85	79.3	6.42	9.65	1.50
1.86	3.35	3.89	79.3	6.49	9.73	1.50
1.87	3.39	3.94	79.3	6.55	9.81	1.50
1.88	3.43	3.98	79.3	6.62	9.89	1.50
1.89	3.47	4.03	79.3	6.68	9.98	1.49
1.90	3.51	4.07	79.3	6.75	10.06	1.49

Age= 58

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.88	2.25	78.9	4.11	6.69	1.63
1.51	1.91	2.29	78.9	4.18	6.77	1.62
1.52	1.95	2.34	78.9	4.24	6.85	1.62
1.53	1.99	2.38	78.9	4.31	6.93	1.61
1.54	2.03	2.42	78.9	4.37	7.02	1.60
1.55	2.07	2.47	78.9	4.44	7.10	1.60
1.56	2.11	2.51	78.9	4.51	7.18	1.59
1.57	2.15	2.56	78.9	4.57	7.26	1.59
1.58	2.19	2.60	78.9	4.64	7.34	1.58
1.59	2.23	2.65	78.9	4.70	7.42	1.58
1.60	2.27	2.69	78.9	4.77	7.51	1.57
1.61	2.31	2.73	78.9	4.84	7.59	1.57
1.62	2.35	2.78	78.9	4.90	7.67	1.56
1.63	2.39	2.82	78.9	4.97	7.75	1.56
1.64	2.43	2.87	78.9	5.03	7.83	1.56
1.65	2.47	2.91	78.9	5.10	7.92	1.55
1.66	2.51	2.96	78.9	5.17	8.00	1.55
1.67	2.55	3.00	78.9	5.23	8.08	1.54
1.68	2.59	3.04	78.9	5.30	8.16	1.54
1.69	2.63	3.09	78.9	5.36	8.24	1.54
1.70	2.67	3.13	78.9	5.43	8.32	1.53
1.71	2.70	3.18	78.9	5.50	8.41	1.53
1.72	2.74	3.22	78.9	5.56	8.49	1.53
1.73	2.78	3.27	78.9	5.63	8.57	1.52
1.74	2.82	3.31	78.9	5.69	8.65	1.52
1.75	2.86	3.35	78.9	5.76	8.73	1.52
1.76	2.90	3.40	78.9	5.83	8.81	1.51
1.77	2.94	3.44	78.9	5.89	8.90	1.51
1.78	2.98	3.49	78.9	5.96	8.98	1.51
1.79	3.02	3.53	78.9	6.02	9.06	1.50
1.80	3.06	3.58	78.9	6.09	9.14	1.50
1.81	3.10	3.62	78.9	6.16	9.22	1.50
1.82	3.14	3.66	78.9	6.22	9.31	1.50
1.83	3.18	3.71	78.9	6.29	9.39	1.49
1.84	3.22	3.75	78.9	6.35	9.47	1.49
1.85	3.26	3.80	78.9	6.42	9.55	1.49
1.86	3.30	3.84	78.9	6.49	9.63	1.49
1.87	3.34	3.89	78.9	6.55	9.71	1.48
1.88	3.38	3.93	78.9	6.62	9.80	1.48
1.89	3.42	3.97	78.9	6.68	9.88	1.48
1.90	3.46	4.02	78.9	6.75	9.96	1.48

Age= 60

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.83	2.20	78.6	4.11	6.59	1.60
1.51	1.86	2.24	78.6	4.18	6.67	1.60
1.52	1.90	2.28	78.6	4.24	6.75	1.59
1.53	1.94	2.33	78.6	4.31	6.84	1.59
1.54	1.98	2.37	78.6	4.37	6.92	1.58
1.55	2.02	2.42	78.6	4.44	7.00	1.58
1.56	2.06	2.46	78.6	4.51	7.08	1.57
1.57	2.10	2.51	78.6	4.57	7.16	1.57
1.58	2.14	2.55	78.6	4.64	7.24	1.56
1.59	2.18	2.59	78.6	4.70	7.33	1.56
1.60	2.22	2.64	78.6	4.77	7.41	1.55
1.61	2.26	2.68	78.6	4.84	7.49	1.55
1.62	2.30	2.73	78.6	4.90	7.57	1.54
1.63	2.34	2.77	78.6	4.97	7.65	1.54
1.64	2.38	2.82	78.6	5.03	7.74	1.54
1.65	2.42	2.86	78.6	5.10	7.82	1.53
1.66	2.46	2.90	78.6	5.17	7.90	1.53
1.67	2.50	2.95	78.6	5.23	7.98	1.53
1.68	2.54	2.99	78.6	5.30	8.06	1.52
1.69	2.58	3.04	78.6	5.36	8.14	1.52
1.70	2.62	3.08	78.6	5.43	8.23	1.51
1.71	2.65	3.13	78.6	5.50	8.31	1.51
1.72	2.69	3.17	78.6	5.56	8.39	1.51
1.73	2.73	3.21	78.6	5.63	8.47	1.51
1.74	2.77	3.26	78.6	5.69	8.55	1.50
1.75	2.81	3.30	78.6	5.76	8.64	1.50
1.76	2.85	3.35	78.6	5.83	8.72	1.50
1.77	2.89	3.39	78.6	5.89	8.80	1.49
1.78	2.93	3.44	78.6	5.96	8.88	1.49
1.79	2.97	3.48	78.6	6.02	8.96	1.49
1.80	3.01	3.52	78.6	6.09	9.04	1.49
1.81	3.05	3.57	78.6	6.16	9.13	1.48
1.82	3.09	3.61	78.6	6.22	9.21	1.48
1.83	3.13	3.66	78.6	6.29	9.29	1.48
1.84	3.17	3.70	78.6	6.35	9.37	1.47
1.85	3.21	3.75	78.6	6.42	9.45	1.47
1.86	3.25	3.79	78.6	6.49	9.53	1.47
1.87	3.29	3.83	78.6	6.55	9.62	1.47
1.88	3.33	3.88	78.6	6.62	9.70	1.47
1.89	3.37	3.92	78.6	6.68	9.78	1.46
1.90	3.41	3.97	78.6	6.75	9.86	1.46

Age= 62

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.78	2.14	78.2	4.11	6.49	1.58
1.51	1.81	2.19	78.2	4.18	6.57	1.57
1.52	1.85	2.23	78.2	4.24	6.66	1.57
1.53	1.89	2.28	78.2	4.31	6.74	1.56
1.54	1.93	2.32	78.2	4.37	6.82	1.56
1.55	1.97	2.36	78.2	4.44	6.90	1.55
1.56	2.01	2.41	78.2	4.51	6.98	1.55
1.57	2.05	2.45	78.2	4.57	7.06	1.55
1.58	2.09	2.50	78.2	4.64	7.15	1.54
1.59	2.13	2.54	78.2	4.70	7.23	1.54
1.60	2.17	2.59	78.2	4.77	7.31	1.53
1.61	2.21	2.63	78.2	4.84	7.39	1.53
1.62	2.25	2.67	78.2	4.90	7.47	1.52
1.63	2.29	2.72	78.2	4.97	7.56	1.52
1.64	2.33	2.76	78.2	5.03	7.64	1.52
1.65	2.37	2.81	78.2	5.10	7.72	1.51
1.66	2.41	2.85	78.2	5.17	7.80	1.51
1.67	2.45	2.90	78.2	5.23	7.88	1.51
1.68	2.49	2.94	78.2	5.30	7.96	1.50
1.69	2.53	2.98	78.2	5.36	8.05	1.50
1.70	2.57	3.03	78.2	5.43	8.13	1.50
1.71	2.60	3.07	78.2	5.50	8.21	1.49
1.72	2.64	3.12	78.2	5.56	8.29	1.49
1.73	2.68	3.16	78.2	5.63	8.37	1.49
1.74	2.72	3.21	78.2	5.69	8.46	1.48
1.75	2.76	3.25	78.2	5.76	8.54	1.48
1.76	2.80	3.29	78.2	5.83	8.62	1.48
1.77	2.84	3.34	78.2	5.89	8.70	1.48
1.78	2.88	3.38	78.2	5.96	8.78	1.47
1.79	2.92	3.43	78.2	6.02	8.86	1.47
1.80	2.96	3.47	78.2	6.09	8.95	1.47
1.81	3.00	3.52	78.2	6.16	9.03	1.47
1.82	3.04	3.56	78.2	6.22	9.11	1.46
1.83	3.08	3.60	78.2	6.29	9.19	1.46
1.84	3.12	3.65	78.2	6.35	9.27	1.46
1.85	3.16	3.69	78.2	6.42	9.36	1.46
1.86	3.20	3.74	78.2	6.49	9.44	1.45
1.87	3.24	3.78	78.2	6.55	9.52	1.45
1.88	3.28	3.83	78.2	6.62	9.60	1.45
1.89	3.32	3.87	78.2	6.68	9.68	1.45
1.90	3.36	3.92	78.2	6.75	9.76	1.45

Age= 64

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.73	2.09	77.8	4.11	6.39	1.56
1.51	1.76	2.14	77.8	4.18	6.48	1.55
1.52	1.80	2.18	77.8	4.24	6.56	1.55
1.53	1.84	2.22	77.8	4.31	6.64	1.54
1.54	1.88	2.27	77.8	4.37	6.72	1.54
1.55	1.92	2.31	77.8	4.44	6.80	1.53
1.56	1.96	2.36	77.8	4.51	6.88	1.53
1.57	2.00	2.40	77.8	4.57	6.97	1.52
1.58	2.04	2.45	77.8	4.64	7.05	1.52
1.59	2.08	2.49	77.8	4.70	7.13	1.52
1.60	2.12	2.53	77.8	4.77	7.21	1.51
1.61	2.16	2.58	77.8	4.84	7.29	1.51
1.62	2.20	2.62	77.8	4.90	7.38	1.50
1.63	2.24	2.67	77.8	4.97	7.46	1.50
1.64	2.28	2.71	77.8	5.03	7.54	1.50
1.65	2.32	2.76	77.8	5.10	7.62	1.49
1.66	2.36	2.80	77.8	5.17	7.70	1.49
1.67	2.40	2.84	77.8	5.23	7.78	1.49
1.68	2.44	2.89	77.8	5.30	7.87	1.48
1.69	2.48	2.93	77.8	5.36	7.95	1.48
1.70	2.52	2.98	77.8	5.43	8.03	1.48
1.71	2.55	3.02	77.8	5.50	8.11	1.48
1.72	2.59	3.07	77.8	5.56	8.19	1.47
1.73	2.63	3.11	77.8	5.63	8.28	1.47
1.74	2.67	3.15	77.8	5.69	8.36	1.47
1.75	2.71	3.20	77.8	5.76	8.44	1.47
1.76	2.75	3.24	77.8	5.83	8.52	1.46
1.77	2.79	3.29	77.8	5.89	8.60	1.46
1.78	2.83	3.33	77.8	5.96	8.68	1.46
1.79	2.87	3.38	77.8	6.02	8.77	1.46
1.80	2.91	3.42	77.8	6.09	8.85	1.45
1.81	2.95	3.46	77.8	6.16	8.93	1.45
1.82	2.99	3.51	77.8	6.22	9.01	1.45
1.83	3.03	3.55	77.8	6.29	9.09	1.45
1.84	3.07	3.60	77.8	6.35	9.18	1.44
1.85	3.11	3.64	77.8	6.42	9.26	1.44
1.86	3.15	3.69	77.8	6.49	9.34	1.44
1.87	3.19	3.73	77.8	6.55	9.42	1.44
1.88	3.23	3.77	77.8	6.62	9.50	1.44
1.89	3.27	3.82	77.8	6.68	9.58	1.43
1.90	3.31	3.86	77.8	6.75	9.67	1.43

Age= 66

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.68	2.04	77.4	4.11	6.30	1.53
1.51	1.71	2.08	77.4	4.18	6.38	1.53
1.52	1.75	2.13	77.4	4.24	6.46	1.52
1.53	1.79	2.17	77.4	4.31	6.54	1.52
1.54	1.83	2.22	77.4	4.37	6.62	1.51
1.55	1.87	2.26	77.4	4.44	6.71	1.51
1.56	1.91	2.30	77.4	4.51	6.79	1.51
1.57	1.95	2.35	77.4	4.57	6.87	1.50
1.58	1.99	2.39	77.4	4.64	6.95	1.50
1.59	2.03	2.44	77.4	4.70	7.03	1.49
1.60	2.07	2.48	77.4	4.77	7.11	1.49
1.61	2.11	2.53	77.4	4.84	7.20	1.49
1.62	2.15	2.57	77.4	4.90	7.28	1.48
1.63	2.19	2.61	77.4	4.97	7.36	1.48
1.64	2.23	2.66	77.4	5.03	7.44	1.48
1.65	2.27	2.70	77.4	5.10	7.52	1.48
1.66	2.31	2.75	77.4	5.17	7.60	1.47
1.67	2.35	2.79	77.4	5.23	7.69	1.47
1.68	2.39	2.84	77.4	5.30	7.77	1.47
1.69	2.43	2.88	77.4	5.36	7.85	1.46
1.70	2.47	2.93	77.4	5.43	7.93	1.46
1.71	2.50	2.97	77.4	5.50	8.01	1.46
1.72	2.54	3.01	77.4	5.56	8.10	1.46
1.73	2.58	3.06	77.4	5.63	8.18	1.45
1.74	2.62	3.10	77.4	5.69	8.26	1.45
1.75	2.66	3.15	77.4	5.76	8.34	1.45
1.76	2.70	3.19	77.4	5.83	8.42	1.45
1.77	2.74	3.24	77.4	5.89	8.50	1.44
1.78	2.78	3.28	77.4	5.96	8.59	1.44
1.79	2.82	3.32	77.4	6.02	8.67	1.44
1.80	2.86	3.37	77.4	6.09	8.75	1.44
1.81	2.90	3.41	77.4	6.16	8.83	1.43
1.82	2.94	3.46	77.4	6.22	8.91	1.43
1.83	2.98	3.50	77.4	6.29	9.00	1.43
1.84	3.02	3.55	77.4	6.35	9.08	1.43
1.85	3.06	3.59	77.4	6.42	9.16	1.43
1.86	3.10	3.63	77.4	6.49	9.24	1.42
1.87	3.14	3.68	77.4	6.55	9.32	1.42
1.88	3.18	3.72	77.4	6.62	9.40	1.42
1.89	3.22	3.77	77.4	6.68	9.49	1.42
1.90	3.26	3.81	77.4	6.75	9.57	1.42

Age= 68

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.63	1.99	77.0	4.11	6.20	1.51
1.51	1.66	2.03	77.0	4.18	6.28	1.50
1.52	1.70	2.08	77.0	4.24	6.36	1.50
1.53	1.74	2.12	77.0	4.31	6.44	1.50
1.54	1.78	2.16	77.0	4.37	6.53	1.49
1.55	1.82	2.21	77.0	4.44	6.61	1.49
1.56	1.86	2.25	77.0	4.51	6.69	1.48
1.57	1.90	2.30	77.0	4.57	6.77	1.48
1.58	1.94	2.34	77.0	4.64	6.85	1.48
1.59	1.98	2.39	77.0	4.70	6.93	1.47
1.60	2.02	2.43	77.0	4.77	7.02	1.47
1.61	2.06	2.47	77.0	4.84	7.10	1.47
1.62	2.10	2.52	77.0	4.90	7.18	1.46
1.63	2.14	2.56	77.0	4.97	7.26	1.46
1.64	2.18	2.61	77.0	5.03	7.34	1.46
1.65	2.22	2.65	77.0	5.10	7.43	1.46
1.66	2.26	2.70	77.0	5.17	7.51	1.45
1.67	2.30	2.74	77.0	5.23	7.59	1.45
1.68	2.34	2.78	77.0	5.30	7.67	1.45
1.69	2.38	2.83	77.0	5.36	7.75	1.45
1.70	2.42	2.87	77.0	5.43	7.83	1.44
1.71	2.45	2.92	77.0	5.50	7.92	1.44
1.72	2.49	2.96	77.0	5.56	8.00	1.44
1.73	2.53	3.01	77.0	5.63	8.08	1.44
1.74	2.57	3.05	77.0	5.69	8.16	1.43
1.75	2.61	3.09	77.0	5.76	8.24	1.43
1.76	2.65	3.14	77.0	5.83	8.32	1.43
1.77	2.69	3.18	77.0	5.89	8.41	1.43
1.78	2.73	3.23	77.0	5.96	8.49	1.42
1.79	2.77	3.27	77.0	6.02	8.57	1.42
1.80	2.81	3.32	77.0	6.09	8.65	1.42
1.81	2.85	3.36	77.0	6.16	8.73	1.42
1.82	2.89	3.40	77.0	6.22	8.82	1.42
1.83	2.93	3.45	77.0	6.29	8.90	1.41
1.84	2.97	3.49	77.0	6.35	8.98	1.41
1.85	3.01	3.54	77.0	6.42	9.06	1.41
1.86	3.05	3.58	77.0	6.49	9.14	1.41
1.87	3.09	3.63	77.0	6.55	9.22	1.41
1.88	3.13	3.67	77.0	6.62	9.31	1.41
1.89	3.17	3.71	77.0	6.68	9.39	1.40
1.90	3.21	3.76	77.0	6.75	9.47	1.40

Age= 70

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.58	1.94	76.7	4.11	6.10	1.48
1.51	1.61	1.98	76.7	4.18	6.18	1.48
1.52	1.65	2.02	76.7	4.24	6.26	1.48
1.53	1.69	2.07	76.7	4.31	6.35	1.47
1.54	1.73	2.11	76.7	4.37	6.43	1.47
1.55	1.77	2.16	76.7	4.44	6.51	1.47
1.56	1.81	2.20	76.7	4.51	6.59	1.46
1.57	1.85	2.25	76.7	4.57	6.67	1.46
1.58	1.89	2.29	76.7	4.64	6.75	1.46
1.59	1.93	2.33	76.7	4.70	6.84	1.45
1.60	1.97	2.38	76.7	4.77	6.92	1.45
1.61	2.01	2.42	76.7	4.84	7.00	1.45
1.62	2.05	2.47	76.7	4.90	7.08	1.44
1.63	2.09	2.51	76.7	4.97	7.16	1.44
1.64	2.13	2.56	76.7	5.03	7.25	1.44
1.65	2.17	2.60	76.7	5.10	7.33	1.44
1.66	2.21	2.64	76.7	5.17	7.41	1.43
1.67	2.25	2.69	76.7	5.23	7.49	1.43
1.68	2.29	2.73	76.7	5.30	7.57	1.43
1.69	2.33	2.78	76.7	5.36	7.65	1.43
1.70	2.37	2.82	76.7	5.43	7.74	1.42
1.71	2.40	2.87	76.7	5.50	7.82	1.42
1.72	2.44	2.91	76.7	5.56	7.90	1.42
1.73	2.48	2.95	76.7	5.63	7.98	1.42
1.74	2.52	3.00	76.7	5.69	8.06	1.42
1.75	2.56	3.04	76.7	5.76	8.15	1.41
1.76	2.60	3.09	76.7	5.83	8.23	1.41
1.77	2.64	3.13	76.7	5.89	8.31	1.41
1.78	2.68	3.18	76.7	5.96	8.39	1.41
1.79	2.72	3.22	76.7	6.02	8.47	1.41
1.80	2.76	3.26	76.7	6.09	8.55	1.40
1.81	2.80	3.31	76.7	6.16	8.64	1.40
1.82	2.84	3.35	76.7	6.22	8.72	1.40
1.83	2.88	3.40	76.7	6.29	8.80	1.40
1.84	2.92	3.44	76.7	6.35	8.88	1.40
1.85	2.96	3.49	76.7	6.42	8.96	1.40
1.86	3.00	3.53	76.7	6.49	9.04	1.39
1.87	3.04	3.57	76.7	6.55	9.13	1.39
1.88	3.08	3.62	76.7	6.62	9.21	1.39
1.89	3.12	3.66	76.7	6.68	9.29	1.39
1.90	3.16	3.71	76.7	6.75	9.37	1.39

Age= 72

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.53	1.88	76.3	4.11	6.00	1.46
1.51	1.56	1.93	76.3	4.18	6.08	1.46
1.52	1.60	1.97	76.3	4.24	6.17	1.45
1.53	1.64	2.02	76.3	4.31	6.25	1.45
1.54	1.68	2.06	76.3	4.37	6.33	1.45
1.55	1.72	2.10	76.3	4.44	6.41	1.44
1.56	1.76	2.15	76.3	4.51	6.49	1.44
1.57	1.80	2.19	76.3	4.57	6.57	1.44
1.58	1.84	2.24	76.3	4.64	6.66	1.44
1.59	1.88	2.28	76.3	4.70	6.74	1.43
1.60	1.92	2.33	76.3	4.77	6.82	1.43
1.61	1.96	2.37	76.3	4.84	6.90	1.43
1.62	2.00	2.41	76.3	4.90	6.98	1.42
1.63	2.04	2.46	76.3	4.97	7.07	1.42
1.64	2.08	2.50	76.3	5.03	7.15	1.42
1.65	2.12	2.55	76.3	5.10	7.23	1.42
1.66	2.16	2.59	76.3	5.17	7.31	1.42
1.67	2.20	2.64	76.3	5.23	7.39	1.41
1.68	2.24	2.68	76.3	5.30	7.47	1.41
1.69	2.28	2.72	76.3	5.36	7.56	1.41
1.70	2.32	2.77	76.3	5.43	7.64	1.41
1.71	2.35	2.81	76.3	5.50	7.72	1.40
1.72	2.39	2.86	76.3	5.56	7.80	1.40
1.73	2.43	2.90	76.3	5.63	7.88	1.40
1.74	2.47	2.95	76.3	5.69	7.97	1.40
1.75	2.51	2.99	76.3	5.76	8.05	1.40
1.76	2.55	3.03	76.3	5.83	8.13	1.40
1.77	2.59	3.08	76.3	5.89	8.21	1.39
1.78	2.63	3.12	76.3	5.96	8.29	1.39
1.79	2.67	3.17	76.3	6.02	8.37	1.39
1.80	2.71	3.21	76.3	6.09	8.46	1.39
1.81	2.75	3.26	76.3	6.16	8.54	1.39
1.82	2.79	3.30	76.3	6.22	8.62	1.39
1.83	2.83	3.34	76.3	6.29	8.70	1.38
1.84	2.87	3.39	76.3	6.35	8.78	1.38
1.85	2.91	3.43	76.3	6.42	8.87	1.38
1.86	2.95	3.48	76.3	6.49	8.95	1.38
1.87	2.99	3.52	76.3	6.55	9.03	1.38
1.88	3.03	3.57	76.3	6.62	9.11	1.38
1.89	3.07	3.61	76.3	6.68	9.19	1.38
1.90	3.11	3.66	76.3	6.75	9.27	1.37

Age= 74

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.48	1.83	75.9	4.11	5.90	1.44
1.51	1.51	1.88	75.9	4.18	5.99	1.43
1.52	1.55	1.92	75.9	4.24	6.07	1.43
1.53	1.59	1.96	75.9	4.31	6.15	1.43
1.54	1.63	2.01	75.9	4.37	6.23	1.42
1.55	1.67	2.05	75.9	4.44	6.31	1.42
1.56	1.71	2.10	75.9	4.51	6.39	1.42
1.57	1.75	2.14	75.9	4.57	6.48	1.42
1.58	1.79	2.19	75.9	4.64	6.56	1.41
1.59	1.83	2.23	75.9	4.70	6.64	1.41
1.60	1.87	2.27	75.9	4.77	6.72	1.41
1.61	1.91	2.32	75.9	4.84	6.80	1.41
1.62	1.95	2.36	75.9	4.90	6.89	1.40
1.63	1.99	2.41	75.9	4.97	6.97	1.40
1.64	2.03	2.45	75.9	5.03	7.05	1.40
1.65	2.07	2.50	75.9	5.10	7.13	1.40
1.66	2.11	2.54	75.9	5.17	7.21	1.40
1.67	2.15	2.58	75.9	5.23	7.29	1.39
1.68	2.19	2.63	75.9	5.30	7.38	1.39
1.69	2.23	2.67	75.9	5.36	7.46	1.39
1.70	2.27	2.72	75.9	5.43	7.54	1.39
1.71	2.30	2.76	75.9	5.50	7.62	1.39
1.72	2.34	2.81	75.9	5.56	7.70	1.39
1.73	2.38	2.85	75.9	5.63	7.79	1.38
1.74	2.42	2.89	75.9	5.69	7.87	1.38
1.75	2.46	2.94	75.9	5.76	7.95	1.38
1.76	2.50	2.98	75.9	5.83	8.03	1.38
1.77	2.54	3.03	75.9	5.89	8.11	1.38
1.78	2.58	3.07	75.9	5.96	8.19	1.38
1.79	2.62	3.12	75.9	6.02	8.28	1.37
1.80	2.66	3.16	75.9	6.09	8.36	1.37
1.81	2.70	3.20	75.9	6.16	8.44	1.37
1.82	2.74	3.25	75.9	6.22	8.52	1.37
1.83	2.78	3.29	75.9	6.29	8.60	1.37
1.84	2.82	3.34	75.9	6.35	8.69	1.37
1.85	2.86	3.38	75.9	6.42	8.77	1.37
1.86	2.90	3.43	75.9	6.49	8.85	1.36
1.87	2.94	3.47	75.9	6.55	8.93	1.36
1.88	2.98	3.51	75.9	6.62	9.01	1.36
1.89	3.02	3.56	75.9	6.68	9.09	1.36
1.90	3.06	3.60	75.9	6.75	9.18	1.36

Age= 76

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.43	1.78	75.5	4.11	5.81	1.41
1.51	1.46	1.82	75.5	4.18	5.89	1.41
1.52	1.50	1.87	75.5	4.24	5.97	1.41
1.53	1.54	1.91	75.5	4.31	6.05	1.40
1.54	1.58	1.96	75.5	4.37	6.13	1.40
1.55	1.62	2.00	75.5	4.44	6.22	1.40
1.56	1.66	2.04	75.5	4.51	6.30	1.40
1.57	1.70	2.09	75.5	4.57	6.38	1.40
1.58	1.74	2.13	75.5	4.64	6.46	1.39
1.59	1.78	2.18	75.5	4.70	6.54	1.39
1.60	1.82	2.22	75.5	4.77	6.62	1.39
1.61	1.86	2.27	75.5	4.84	6.71	1.39
1.62	1.90	2.31	75.5	4.90	6.79	1.38
1.63	1.94	2.35	75.5	4.97	6.87	1.38
1.64	1.98	2.40	75.5	5.03	6.95	1.38
1.65	2.02	2.44	75.5	5.10	7.03	1.38
1.66	2.06	2.49	75.5	5.17	7.11	1.38
1.67	2.10	2.53	75.5	5.23	7.20	1.38
1.68	2.14	2.58	75.5	5.30	7.28	1.37
1.69	2.18	2.62	75.5	5.36	7.36	1.37
1.70	2.22	2.67	75.5	5.43	7.44	1.37
1.71	2.25	2.71	75.5	5.50	7.52	1.37
1.72	2.29	2.75	75.5	5.56	7.61	1.37
1.73	2.33	2.80	75.5	5.63	7.69	1.37
1.74	2.37	2.84	75.5	5.69	7.77	1.36
1.75	2.41	2.89	75.5	5.76	7.85	1.36
1.76	2.45	2.93	75.5	5.83	7.93	1.36
1.77	2.49	2.98	75.5	5.89	8.01	1.36
1.78	2.53	3.02	75.5	5.96	8.10	1.36
1.79	2.57	3.06	75.5	6.02	8.18	1.36
1.80	2.61	3.11	75.5	6.09	8.26	1.36
1.81	2.65	3.15	75.5	6.16	8.34	1.36
1.82	2.69	3.20	75.5	6.22	8.42	1.35
1.83	2.73	3.24	75.5	6.29	8.51	1.35
1.84	2.77	3.29	75.5	6.35	8.59	1.35
1.85	2.81	3.33	75.5	6.42	8.67	1.35
1.86	2.85	3.37	75.5	6.49	8.75	1.35
1.87	2.89	3.42	75.5	6.55	8.83	1.35
1.88	2.93	3.46	75.5	6.62	8.91	1.35
1.89	2.97	3.51	75.5	6.68	9.00	1.35
1.90	3.01	3.55	75.5	6.75	9.08	1.34

Age= 78

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.38	1.73	75.1	4.11	5.71	1.39
1.51	1.41	1.77	75.1	4.18	5.79	1.39
1.52	1.45	1.82	75.1	4.24	5.87	1.38
1.53	1.49	1.86	75.1	4.31	5.95	1.38
1.54	1.53	1.90	75.1	4.37	6.04	1.38
1.55	1.57	1.95	75.1	4.44	6.12	1.38
1.56	1.61	1.99	75.1	4.51	6.20	1.38
1.57	1.65	2.04	75.1	4.57	6.28	1.37
1.58	1.69	2.08	75.1	4.64	6.36	1.37
1.59	1.73	2.13	75.1	4.70	6.44	1.37
1.60	1.77	2.17	75.1	4.77	6.53	1.37
1.61	1.81	2.21	75.1	4.84	6.61	1.37
1.62	1.85	2.26	75.1	4.90	6.69	1.36
1.63	1.89	2.30	75.1	4.97	6.77	1.36
1.64	1.93	2.35	75.1	5.03	6.85	1.36
1.65	1.97	2.39	75.1	5.10	6.94	1.36
1.66	2.01	2.44	75.1	5.17	7.02	1.36
1.67	2.05	2.48	75.1	5.23	7.10	1.36
1.68	2.09	2.52	75.1	5.30	7.18	1.36
1.69	2.13	2.57	75.1	5.36	7.26	1.35
1.70	2.17	2.61	75.1	5.43	7.34	1.35
1.71	2.20	2.66	75.1	5.50	7.43	1.35
1.72	2.24	2.70	75.1	5.56	7.51	1.35
1.73	2.28	2.75	75.1	5.63	7.59	1.35
1.74	2.32	2.79	75.1	5.69	7.67	1.35
1.75	2.36	2.83	75.1	5.76	7.75	1.35
1.76	2.40	2.88	75.1	5.83	7.83	1.34
1.77	2.44	2.92	75.1	5.89	7.92	1.34
1.78	2.48	2.97	75.1	5.96	8.00	1.34
1.79	2.52	3.01	75.1	6.02	8.08	1.34
1.80	2.56	3.06	75.1	6.09	8.16	1.34
1.81	2.60	3.10	75.1	6.16	8.24	1.34
1.82	2.64	3.14	75.1	6.22	8.33	1.34
1.83	2.68	3.19	75.1	6.29	8.41	1.34
1.84	2.72	3.23	75.1	6.35	8.49	1.34
1.85	2.76	3.28	75.1	6.42	8.57	1.34
1.86	2.80	3.32	75.1	6.49	8.65	1.33
1.87	2.84	3.37	75.1	6.55	8.73	1.33
1.88	2.88	3.41	75.1	6.62	8.82	1.33
1.89	2.92	3.45	75.1	6.68	8.90	1.33
1.90	2.96	3.50	75.1	6.75	8.98	1.33

Age= 80

Height (m)	FEV_1 (*L*)	FVC (*L*)	Ratio (%)	TLC (*L*)	DLco (ml min^{-1} $mmHg^{-1}$)	Kco (ml min^{-1} $mmHg^{-1}L^{-1}$)
1.50	1.33	1.68	74.8	4.11	5.61	1.36
1.51	1.36	1.72	74.8	4.18	5.69	1.36
1.52	1.40	1.76	74.8	4.24	5.77	1.36
1.53	1.44	1.81	74.8	4.31	5.86	1.36
1.54	1.48	1.85	74.8	4.37	5.94	1.36
1.55	1.52	1.90	74.8	4.44	6.02	1.36
1.56	1.56	1.94	74.8	4.51	6.10	1.35
1.57	1.60	1.99	74.8	4.57	6.18	1.35
1.58	1.64	2.03	74.8	4.64	6.26	1.35
1.59	1.68	2.07	74.8	4.70	6.35	1.35
1.60	1.72	2.12	74.8	4.77	6.43	1.35
1.61	1.76	2.16	74.8	4.84	6.51	1.35
1.62	1.80	2.21	74.8	4.90	6.59	1.34
1.63	1.84	2.25	74.8	4.97	6.67	1.34
1.64	1.88	2.30	74.8	5.03	6.76	1.34
1.65	1.92	2.34	74.8	5.10	6.84	1.34
1.66	1.96	2.38	74.8	5.17	6.92	1.34
1.67	2.00	2.43	74.8	5.23	7.00	1.34
1.68	2.04	2.47	74.8	5.30	7.08	1.34
1.69	2.08	2.52	74.8	5.36	7.16	1.34
1.70	2.12	2.56	74.8	5.43	7.25	1.33
1.71	2.15	2.61	74.8	5.50	7.33	1.33
1.72	2.19	2.65	74.8	5.56	7.41	1.33
1.73	2.23	2.69	74.8	5.63	7.49	1.33
1.74	2.27	2.74	74.8	5.69	7.57	1.33
1.75	2.31	2.78	74.8	5.76	7.66	1.33
1.76	2.35	2.83	74.8	5.83	7.74	1.33
1.77	2.39	2.87	74.8	5.89	7.82	1.33
1.78	2.43	2.92	74.8	5.96	7.90	1.33
1.79	2.47	2.96	74.8	6.02	7.98	1.33
1.80	2.51	3.00	74.8	6.09	8.06	1.32
1.81	2.55	3.05	74.8	6.16	8.15	1.32
1.82	2.59	3.09	74.8	6.22	8.23	1.32
1.83	2.63	3.14	74.8	6.29	8.31	1.32
1.84	2.67	3.18	74.8	6.35	8.39	1.32
1.85	2.71	3.23	74.8	6.42	8.47	1.32
1.86	2.75	3.27	74.8	6.49	8.55	1.32
1.87	2.79	3.31	74.8	6.55	8.64	1.32
1.88	2.83	3.36	74.8	6.62	8.72	1.32
1.89	2.87	3.40	74.8	6.68	8.80	1.32
1.90	2.91	3.45	74.8	6.75	8.88	1.32

Letter to the Editor

Choice of Anticoagulant Critically Affects Measurement of Circulating Platelet-Leukocyte Complexes

Stylianos Bournazos, Jillian Rennie, Simon P. Hart, Ian Dransfield

In circulation, platelets adhere to leukocytes forming relatively stable complexes that have been reported to be elevated in cases of unstable angina, myocardial infarction, coronary artery disease, and postangioplasty restenosis.[1–10] For this reason, measurement of circulating platelet-leukocyte complexes has been proposed as an early and accurate marker of in vivo platelet activation and myocardial injury after infarction.[9,10] Increased levels of such complexes have also been noted in a range of chronic inflammatory diseases, including rheumatoid arthritis, end-stage renal failure, type I diabetes, and systemic lupus erythematosus.[11–13]

In the majority of published studies that have examined platelet-monocyte or platelet-polymorphonuclear (PMN) leukocyte complexes in human peripheral venous blood, sodium citrate (0.32 to 0.38%), a calcium-depleting agent, has been used as the blood anticoagulant. Because platelet adhesion to leukocytes is predominantly mediated by calcium-dependent interactions between platelet P-selectin and its leukocyte counter-receptor, P-selectin glycoprotein ligand-1 (PSGL-1),[14] we aimed to determine whether calcium depletion by sodium citrate could affect platelet-leukocyte complex formation.

For this reason, platelet-monocyte and -PMN leukocyte complexes in citrated blood (0.38% wt/vol final concentration) were measured using 2-color flow cytometry (described in[3]) and compared with those observed in blood anticoagulated by hirudin (200 U mL^{-1}; lepirudin), heparin (10 U mL^{-1}), or PPACK (D-phenylalanyl-L-propyl-L-arginine chloromethylketone; 75 μmol/L), which act independently of calcium chelation.

We observed significantly lower percentages of platelet binding to monocytes (defined as $CD42a^+/CD14^+$ events) in blood anticoagulated with sodium citrate compared with hirudin-, heparin-, or PPACK-anticoagulated blood (Figure). No significant differences in PMN-platelet binding were evident using the different anticoagulants (13.7% ±2.3 for citrate, 18.8% ±5 for heparin, 16.8% ±4.4 for hirudin, 19.3% ±4.3 for PPACK; n=6), an observation reflecting lower levels of cation-dependent platelet adhesion that might be attributed to lower levels of PSGL-1 expression on PMN leukocytes compared with monocytes (data not shown).

Incubation of blood with EDTA (10 mmol/L) resulted in a substantial decrease (to approximately 15%) in monocyte-platelet binding, an effect that was irrespective of the anticoagulant used (Figure). Because EDTA and sodium citrate are both calcium chelators but EDTA acts on other divalent cations as well, one interpretation could be the involvement of other divalent cation-dependent interactions (eg, Mg^{2+}-dependent integrin-mediated interactions). However, use of EGTA, a selective chelator of Ca^{2+}, resulted in comparable monocyte-platelet binding to that observed in EDTA (14.3% ±1.6 for EDTA; 14.1% ±1.4 for EGTA; n=4). We therefore suggest that the higher percentage of monocytes binding to platelets observed in blood anticoagulated with citrate compared with EDTA reflects the relative inefficiency of sodium citrate as calcium chelator.

We conclude that in the presence of sodium citrate, a substantial component of the Ca^{2+}-dependent interactions between platelets and monocytes is excluded, hence leading to underestimation of the actual percentage of monocyte-platelet binding in circulation. When compared with EDTA, sodium citrate has a limited capacity for calcium depletion and as a consequence, residual Ca^{2+} ions could influence platelet adhesion to monocytes. The existence of divalent cation-dependent and -independent mechanisms for platelet adhesion to monocytes is well established.[3] We suggest that previous studies describing an association of platelet-monocyte binding with disease pathogenesis should be carefully reexamined because the contribution of these component interactions cannot be precisely determined when citrate was used as blood anticoagulant.

In summary, without necessarily proposing the use of one anticoagulant in preference to another, our findings clearly indicate that the choice of blood anticoagulation is critically important in studies of platelet-leukocyte interactions. Particular caution is required when interpreting and comparing results from studies that have made use of different anticoagulants.

Disclosures

None.

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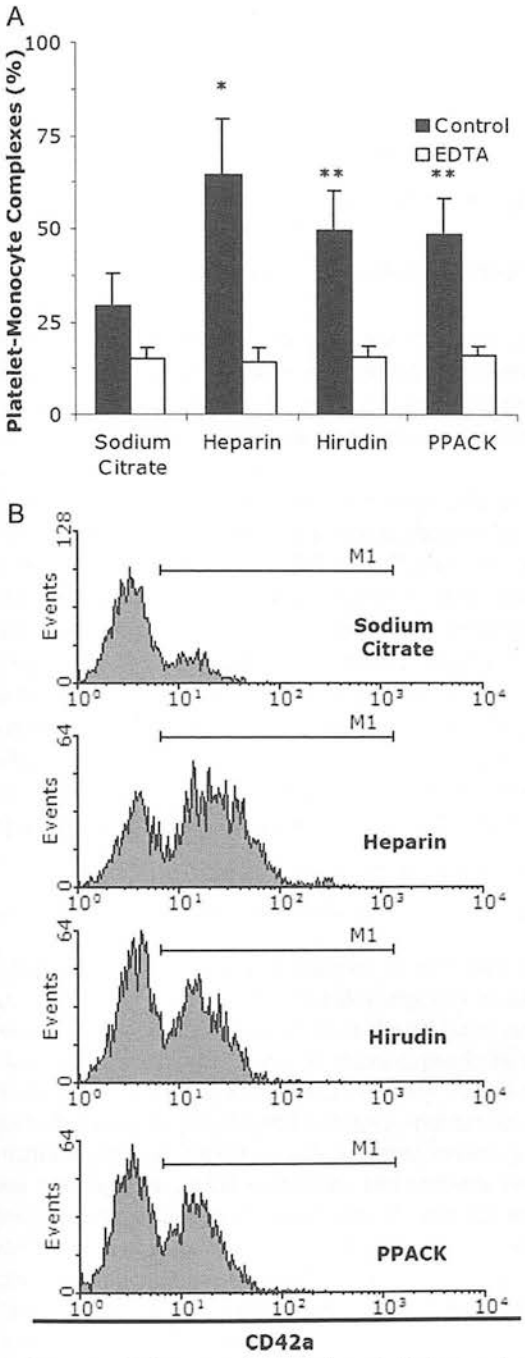

Figure. Impact of different anticoagulation strategies on the percentage of platelet-monocyte complexes. Peripheral venous blood obtained from 6 healthy donors was anticoagulated with: sodium citrate (0.38% wt/vol), heparin (10 U ml^{-1}), hirudin (200 U ml^{-1}), or PPACK (75 μmol/L) and incubated with □ or without ■ 10 mmol/L EDTA. Using 2-color flow cytometry, monocytes ($CD14^+$) were identified and the percentage of platelet binding to them (defined as $CD14^+/CD42a^+$ events) was measured. A, In sodium citrate anticoagulated blood, significantly lower percentages of platelet-monocyte complexes were observed compared with blood anticoagulated with heparin, hirudin, or PPACK. Incubation of blood with EDTA (10 mmol/L) resulted in all cases in a substantial decrease in platelet-monocyte complexes. Results are presented as mean±SD from 6 independent experiments in which platelet-monocyte complexes were measured in different donors. *$P<0.01$; **$P<0.05$, compared with sodium citrate. B, Representative flow cytometry histograms of monocytes ($CD42a^-$) and platelet-monocyte complexes ($CD42a^+$; M1 region) in blood anticoagulated with sodium citrate, heparin, hirudin, or PPACK. Histograms are gated only on $CD14^+$ cells.

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Monocyte Functional Responsiveness After PSGL-1–Mediated Platelet Adhesion Is Dependent on Platelet Activation Status

Stylianos Bournazos, Jillian Rennie, Simon P. Hart, Keith A.A. Fox, Ian Dransfield

Objective—Acute coronary diseases are characterized by elevated levels of circulating platelet-leukocyte complexes, raising the possibility that proinflammatory processes might be initiated in leukocytes after platelet adhesion. Here we examined the mechanism of platelet binding to polymorphonuclear leukocytes, monocytes, and monocyte subsets and investigated the potential functional consequences of monocyte binding to minimally activated or thrombin-activated platelets.

Methods and Results—In this article, we describe key differences in terms of stability of PSGL-1–mediated interaction of platelets with monocytes and polymorphonuclear leukocytes and a small but significant difference in platelet binding to monocyte subsets ($CD14^{high}$ and $CD14^{low}$/HLA-DR^{high}). We also report differential effects of platelet binding on monocyte functional responses between minimally and thrombin-activated platelets. In particular, monocyte CD11b expression and release of proinflammatory cytokines, like interleukin 1β and tumor necrosis factor α, were significantly upregulated on adhesion of stimulated platelets, whereas unstimulated platelets had no effect. Moreover, binding of unstimulated, but not of thrombin-activated, platelets to monocytes had no impact on NF-κB activity, monocyte migration, and induction of apoptosis in the absence of survival factors.

Conclusions—Our data suggest that in the absence of overt activation, PSGL-1–P-selectin–dependent platelet binding to monocytes represents a normal physiological process with little impact on the potential of monocytes to cause vascular injury. (***Arterioscler Thromb Vasc Biol.* 2008;28:1491-1498**)

Key Words: monocyte ■ platelet ■ adhesion ■ selectin ■ thrombin ■ proinflammatory

Adhesion of platelets and leukocytes to activated endothelium is an early event in the development of atherosclerosis.[1] Activated platelets deposit at the damaged arterial wall associated with unstable plaque rupture, precipitating or potentiating thrombus formation and coronary vascular obstruction. In addition, platelet and leukocyte interactions with endothelium play an important role in acute coronary syndromes (ACS), myocardial infarction, and unstable angina. Whereas current strategies for treatment of ACS are mainly targeted to limit platelet aggregation via glycoprotein IIb-IIIa antagonists or thienopyridines, there is evidence that alternative selectin-dependent adhesion pathways are also important in the development of vascular injury.[2]

Binding of platelets to leukocytes can be demonstrated in whole blood samples from healthy volunteers, and the proportion of platelet-bound leukocytes is elevated in cases of unstable angina, myocardial infarction, coronary artery disease, and postangioplasty restenosis.[3–5] Also, increased levels of platelet–monocyte complexes were noted in patients with type 2 diabetes,[6,7] end stage renal disease,[8] and rheumatoid arthritis[9] and in smokers.[10] We have previously shown that platelets bind to monocytes predominantly via a divalent cation-dependent P-selectin–P-selectin glycoprotein ligand-1 (PSGL-1/CD162) pathway. In addition, residual divalent cation-independent platelet-monocyte binding indicates that alternative molecular mechanisms for interaction also exist.[5] Antibody-mediated cross-linking induces association of PSGL-1 with cytoskeletal proteins including ezrin and also signaling cascades eg, Syk tyrosine kinases.[11,12] Because PSGL-1 may be induced to redistribute after binding of platelets to leukocytes, engagement of PSGL-1 has the potential to influence leukocyte behavior through signaling pathways or via cytoskeletal regulation.

Based on the proinflammatory signaling cascades after PSGL-1 engagement, as well as the association of platelet-monocyte complexes with ACS, increased platelet-monocyte binding was suggested to represent a risk factor for development of atherosclerosis,[13,14] possibly as a consequence of altered leukocyte recruitment and activation status. However, there is little published evidence for priming and activation of

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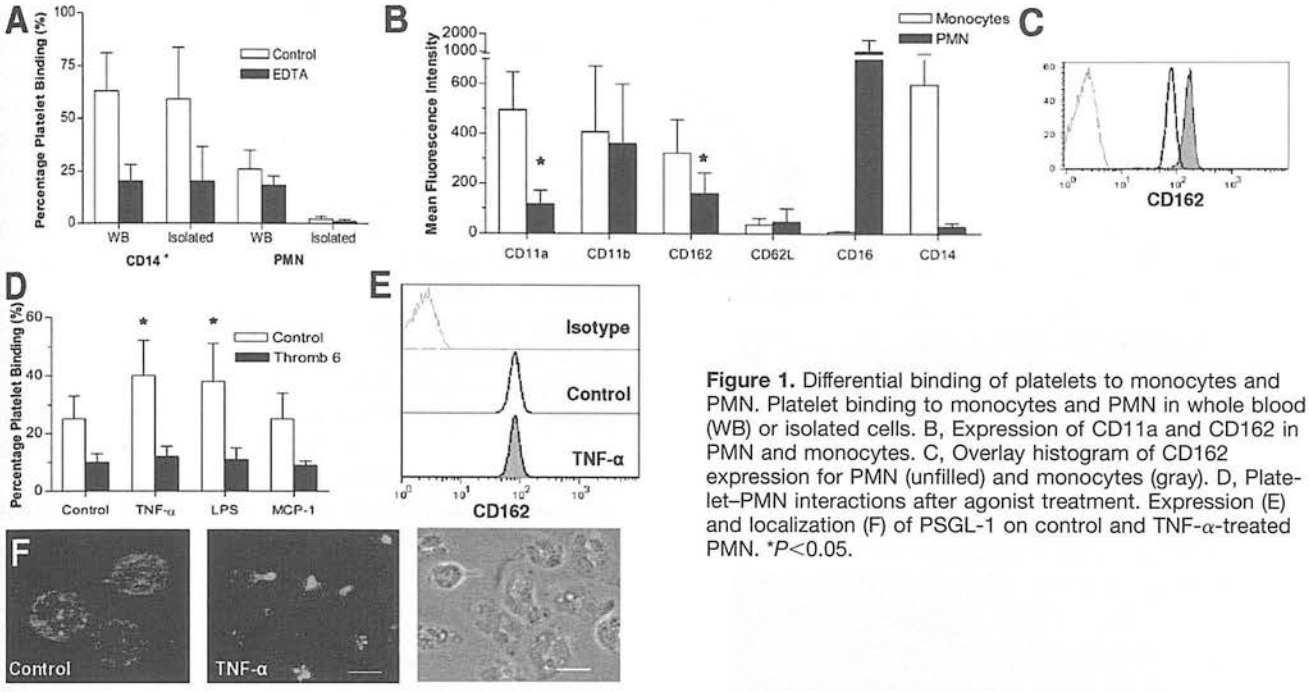

Figure 1. Differential binding of platelets to monocytes and PMN. Platelet binding to monocytes and PMN in whole blood (WB) or isolated cells. B, Expression of CD11a and CD162 in PMN and monocytes. C, Overlay histogram of CD162 expression for PMN (unfilled) and monocytes (gray). D, Platelet–PMN interactions after agonist treatment. Expression (E) and localization (F) of PSGL-1 on control and TNF-α-treated PMN. *$P<0.05$.

peripheral blood polymorphonuclear leukocytes (PMN) or monocytes that would be consistent with a former platelet-bound population.[15] Thus, although platelets can be demonstrated to bind to leukocytes in whole blood, this binding may not necessarily influence leukocyte function in vitro.

Here, we examined the differences in the regulation of platelet adhesion to PMN and monocytes and analyzed platelet binding to monocyte subsets ($CD14^{high}$ and $CD14^{low}$/$HLA\text{-}DR^{high}$). We also investigated the functional consequences of platelet binding to monocytes in terms of cell surface receptor expression, cytokine production, cell migration, activation of proinflammatory transcription factors, and engagement of apoptotic programs.

Methods

For detailed descriptions of the Materials and Methods and Figure Legends, please see http://atvb.ahajournals.org.

Immunolabeling and Flow Cytometry

Leukocyte-platelet interactions were determined using fluorochrome conjugated anti-CD42a mAb as described.[5] Flow cytometric analysis of the samples was performed using a BD FACSCalibur or FACScan cytometer.

Cytokine Measurement

Monocytes (with or without platelets/agonists) were incubated at 37°C for 5 hours, and cytokines (IL-1β and TNF-α) were measured using a fluorescent bead-based sandwich assay (BD cytometric bead array). Analysis of the samples was performed using a BD FACS Array Bioanalysis System.

Transmigration Assay

Monocytes were preincubated with or without platelets, and transwell migration (1 to 6 hours; 37°C) in response to 6.25 ng ml^{-1} complement C5a was measured as described.[16]

Immunoblotting

Monocytes after treatment with agonists or platelets (30 minutes, 37°C) were lysed, and proteins were resolved by SDS-PAGE. For IκBα detection, rabbit monoclonal anti-IκBα (1:2500; E130, Abcam) was used followed by HRP-conjugated goat antirabbit IgG (1:2500; Dako Cytomation).

Results

Preferential Binding of Platelets to Circulating Monocytes Over PMN Cells

The role of P-selectin–PSGL-1 interaction in platelet binding to monocytes and PMN was determined using either function blocking antibodies against PSGL-1 (PL-1) or EDTA to chelate divalent cations. For monocytes, addition of EDTA resulted in a substantial decrease in platelet adhesion, indicating a divalent cation dependency (supplemental Figures I and II, available online at http://atvb.ahajournals.org). Consistent with our previous data indicating a major role for PSGL-1 in mediating platelet-monocyte binding,[5] function-blocking anti–PSGL-1 antibodies caused a similar inhibition of platelet binding to addition of EDTA (supplemental Figures I and II). Platelet adhesion to PMN was also inhibited by divalent cation chelation or PSGL-1 inhibition, but to a lesser extent, an observation that might reflect the lower basal levels of platelet adhesion (supplemental Figure II).

The importance of PSGL-1–P-selectin interaction in mediating platelet adhesion to leukocytes was further investigated by examining the effect of platelet activators on the formation of platelet-leukocyte complexes. Platelet activation with either thrombin (1 U ml^{-1}) or TRAP (20 μmol/L) significantly increased platelet binding to both monocytes and PMN in a divalent cation-dependent manner (supplemental Figure III), an effect that was paralleled by increased expression of platelet P-selectin (supplemental Figure IV).

Although platelet adhesion to both monocytes and PMN has been shown to be mediated primarily through PSGL-1-P-selectin interaction, we observed a profound difference in the extent of platelet adhesion to these 2 leukocyte cell types

in whole blood samples (supplemental Figure II). Notably, comparison of the extent of platelet binding either in unfractionated whole blood samples or after density gradient cell separation procedures revealed differences in the stability of platelet adhesion to monocytes compared to PMN (Figure 1A). There was no difference in the proportion of monocytes that had bound platelets in whole blood and mononuclear cell fractions, and binding exhibited similar divalent cation dependency. In contrast, while platelet binding to PMN could be detected in whole blood, very low levels of platelet binding were observed in isolated PMN cell preparations, despite following similar isolation procedures as in monocytes. Interestingly, divalent cation-independent platelet binding to density gradient separated PMN cells was virtually abolished in the presence of EDTA.

We examined a panel of adhesion receptor molecules on monocytes and PMN from whole blood samples using flow cytometric analysis to determine whether the observed differential stability of platelet-PMN and platelet-monocyte binding reflected differences in the levels of surface expression of PSGL-1. No significant differences in the expression of CD62L or CD11b were apparent between monocytes and PMN (Figure 1B). In contrast, significantly lower levels of CD11a and PSGL-1 (CD162) were found for PMN when compared to monocytes (Figure 1B and 1C). Based on our finding that platelet-leukocyte binding displays comparable sensitivity to EGTA (a specific Ca^{2+} chelator) and EDTA[17] and sensitivity to PSGL-1 blockade, one implication of the above data are that the levels of PSGL-1 expression might determine the extent of platelet-leukocyte interactions. However, additional experiments demonstrated that increased platelet binding to PMN occurred after exposure to TNF-α or lipopolysaccharide (LPS) without affecting PMN expression of PSGL-1, suggesting that platelet binding could be regulated independently of receptor expression (Figure 1D and 1E). We therefore examined PSGL-1 localization in nonactivated or TNF-α–activated PMN (10 ng ml^{-1}; 45 minutes, 37°C) by immunofluorescence microscopy. Whereas PSGL-1 is evenly distributed throughout the entire surface of nonactivated PMN (Figure 1F), it becomes localized in uropods of TNF-α–activated PMN, suggesting that receptor localization might contribute to the regulation of platelet binding.

As platelet-monocyte binding was found to be more resistant to dissociation during cell isolation than platelet-PMN binding, we next examined the stability of platelet adhesion on monocytes after brief (30 seconds) vortexing of samples in the presence or absence of divalent cation chelators (Figure 2A). Repeated vortexing of platelet-monocyte conjugates in the presence of divalent cations had little impact on the proportion of monocytes with bound platelets. In contrast, vortexing in the presence of EDTA readily reversed binding. Similar results were obtained when P-selectin–PSGL-1 interactions were disrupted with function-blocking antibodies (data not shown). Platelet binding was further examined using fluorescent labeled platelets isolated using minimal activation protocols and "platelet-free" monocytes separated by immunomagnetic selection techniques in the presence of EDTA. Maximal binding of platelets to monocytes occurred within 15 minutes of coincubation and

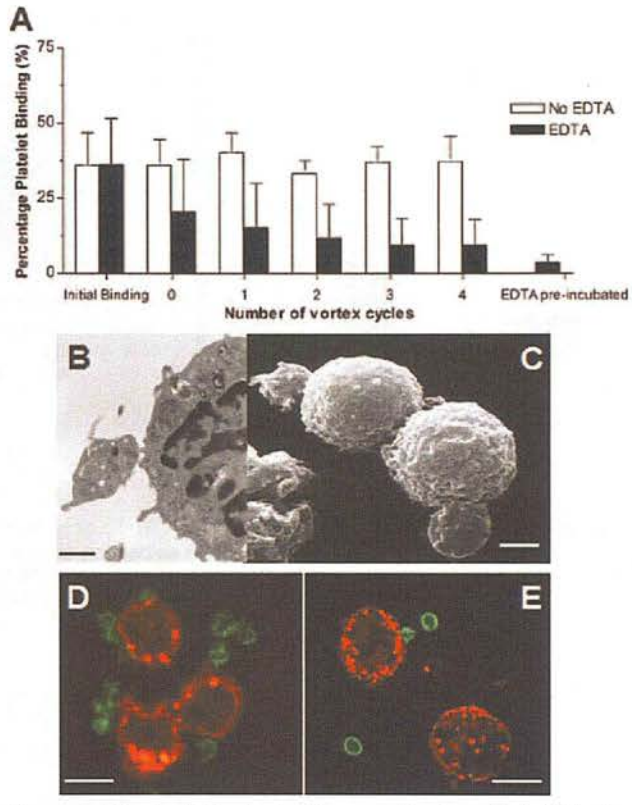

Figure 2. Effects of divalent cations on binding of platelets to monocytes. A, Platelet-monocyte complexes after vortexing with (■) or without (□) EDTA. Transmission (B) and scanning (C) electron microscopy analysis of platelet-monocyte complexes in the presence of divalent cations. Binding of intact platelets (CD42a:FITC) to monocytes (CD14:PE) with (D) and without divalent cations (E).

was dependent on the number of platelets added back, suggesting that binding can occur relatively quickly (data not shown).

Examination of platelet-monocyte interactions by transmission and scanning electron microscopy in the presence of divalent cations revealed that most platelet binding was to membrane projections (Figure 2B and 2C), although close apposition of the platelet membrane to the monocyte surface was also observed in some cases (data not shown). The possibility that in the absence of divalent cations platelet microparticles rather than intact platelets were bound to monocytes was excluded by examination of platelet-monocyte binding using scanning laser confocal microscopy (Figure 2D and 2E). Our analysis clearly showed that intact platelets bind to monocytes with little evidence of microparticle binding.

Measurement of Platelet Adhesion to Monocyte Subsets

It is now well established that there are 2 distinct subpopulations of monocytes that can be defined in terms of patterns of expression of CD14, CD16, and HLA-DR.[18] Using 3-color flow cytometry, the patterns of platelet binding to these different monocyte populations were defined in the presence or absence of EDTA (Figure 3A and 3B). In paired analysis (n=13), we found a small but significant difference in the

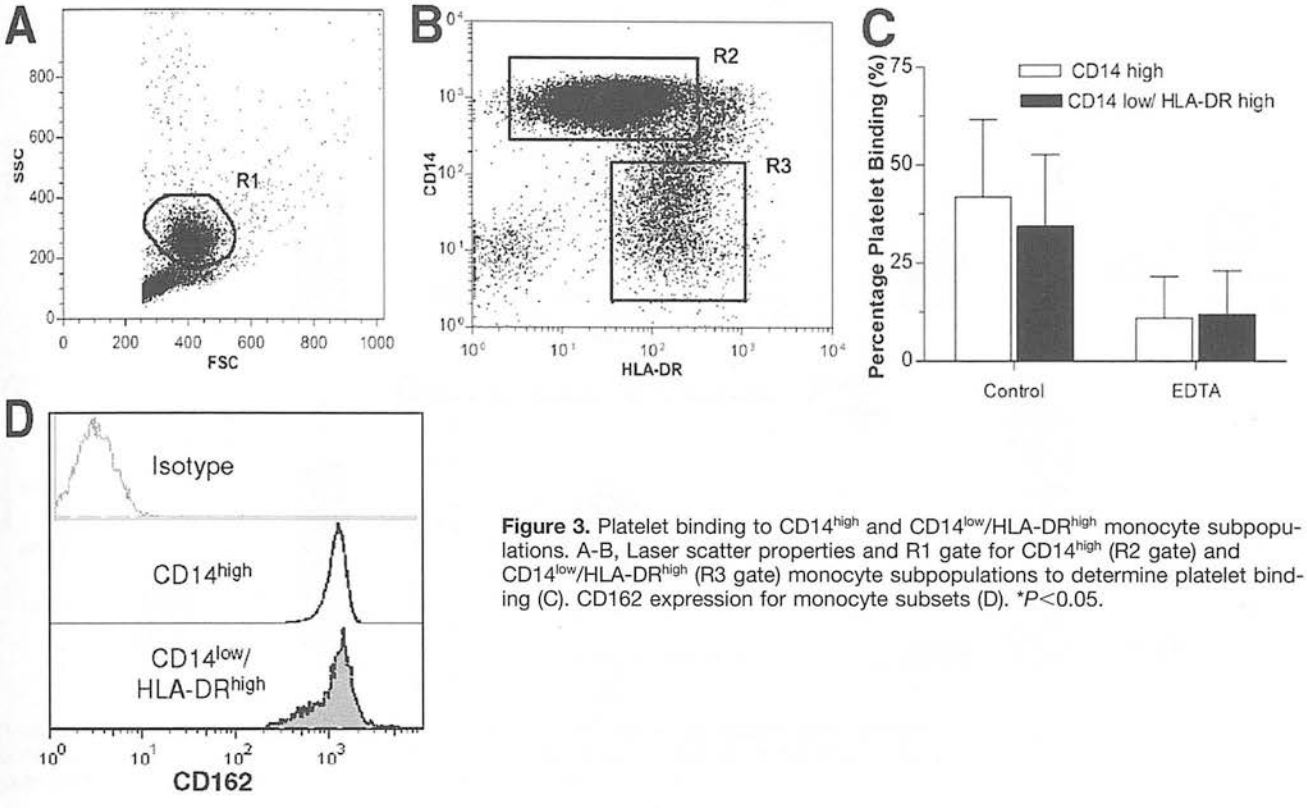

Figure 3. Platelet binding to $CD14^{high}$ and $CD14^{low}/HLA\text{-}DR^{high}$ monocyte subpopulations. A-B, Laser scatter properties and R1 gate for $CD14^{high}$ (R2 gate) and $CD14^{low}/HLA\text{-}DR^{high}$ (R3 gate) monocyte subpopulations to determine platelet binding (C). CD162 expression for monocyte subsets (D). $^*P<0.05$.

extent of platelet binding to the $CD14^{low}CD16^{low}HLA\text{-}DR^{high}$ subset when compared with the $CD14^{high}$ expressing monocytes (Figure 3C), even though PSGL-1 expression between these subsets was at similar levels (Figure 3D). In the presence of EDTA, the levels of binding of platelets to both monocyte subsets were equivalent suggesting similar divalent cation sensitivity.

Functional Effects of Platelet Adhesion to Monocytes

Platelet adhesion to monocytes represents a more stable and long-lived interaction compared to PMN, and thus it is possible that these interactions could influence monocyte function. We therefore wished to investigate the impact of PSGL-1 engagement on monocyte functional activity after binding of unstimulated and TRAP-activated platelets. We measured the expression of CD62L (rapidly shed on cell activation) and CD11b (which is mobilized from intracellular stores) on the surface of monocytes with or without bound platelets as early markers of monocyte activation. Three-color flow cytometric analysis of whole blood samples failed to reveal a significant effect of platelet binding on surface expression of either CD11b or CD62L in the absence of overt activation, with similar levels of receptor expression on CD42a positive (platelet-bound) and negative (platelet-free) monocytes (Figure 4A through 4D). Consistent with this observation, there were no significant differences in monocyte transwell migration in response to C5a for monocytes with or without bound unstimulated platelets (supplemental Figure V). This observation suggests that binding of unstimulated platelets fail to influence monocyte adhesion and cytoskeletal reorganization, which is required for efficient migration. Surprisingly, no significant change in the expression of CD62L was noted for monocytes that had bound TRAP-activated platelets. In contrast, CD11b expression was substantially increased for monocytes that had bound TRAP-activated platelets consistent with an effect of adhesion of activated (but not unstimulated) platelets on monocyte functional responses (Figure 4A and 4C).

We next measured cytokine release from monocytes coincubated with unstimulated or thrombin-stimulated platelets (37°C, 5 hours). Interestingly, monocyte expression and release of IL-1β and TNF-α was substantially upregulated for monocytes coincubated with thrombin-stimulated platelets (Figure 4E and 4F), but not in unstimulated platelets. Binding of thrombin-activated platelets has been previously shown to activate NF-κB,[19] a critical regulator of proinflammatory gene expression and a known survival factor for myeloid cells.[20] We therefore examined whether monocytes with bound platelets displayed translocation of NF-κB to the nucleus, comparing the effects of unstimulated and thrombin-stimulated platelets. Whereas monocytes with minimally activated platelets bound showed cytoplasmic localization of NF-κB, monocytes with thrombin-activated platelets showed nuclear translocation of NF-κB (Figure 5A). IκBα was detectable in the cytoplasm of monocytes with unstimulated platelets (Figure 5B), but decreased cytoplasmic expression was noted when thrombin-stimulated platelets were bound (Figure 5B), suggestive of IκB degradation. Quantification of monocyte IκBα expression by immunoblot analysis revealed that binding of unstimulated platelets had no impact on IκBα expression when compared with untreated monocytes alone (Figure 5C).

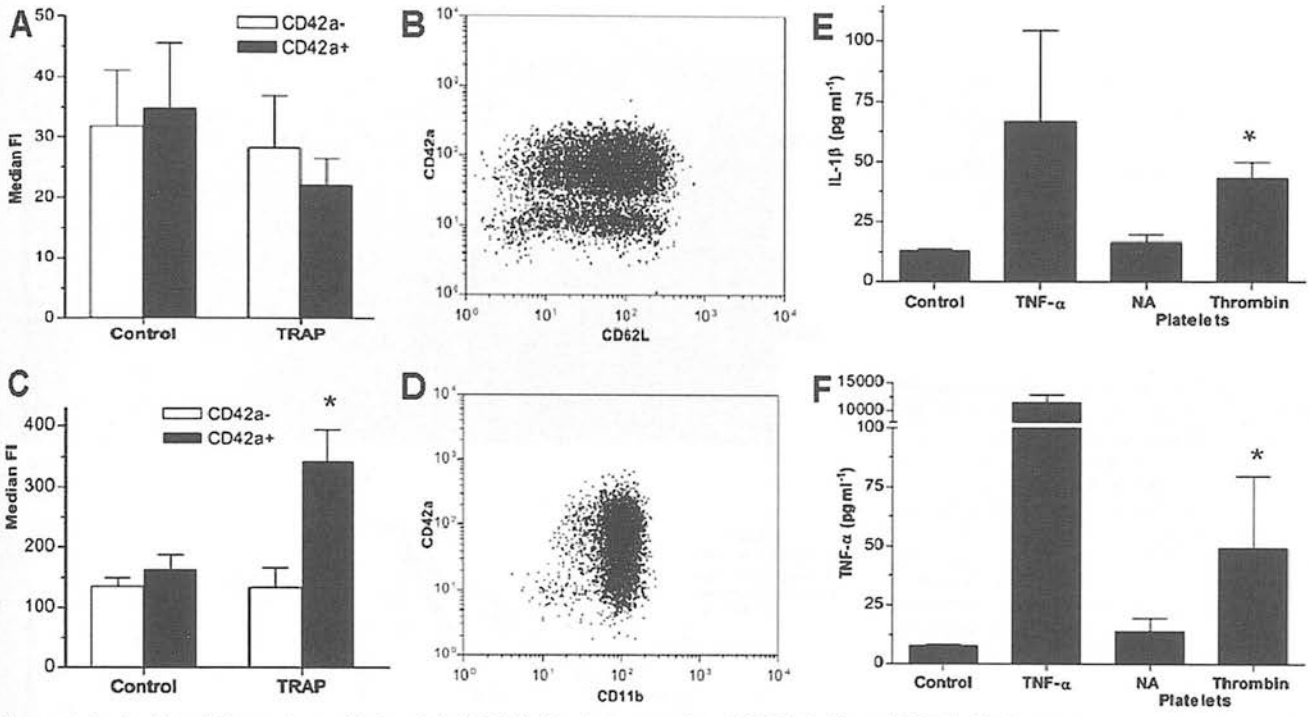

Figure 4. Lack of proinflammatory effects of platelet binding to monocytes. CD62L (A-B) and CD11b (C-D) expression for $CD42a^+$ and $CD42a^-$ monocytes in unstimulated or TRAP-stimulated whole blood samples. IL-1β (E) and TNF-α (F) release from monocytes coincubated with unstimulated (NA) or thrombin-stimulated platelets. *$P<0.05$.

Similarly, unstimulated platelet binding also failed to affect monocyte apoptotic programs again suggesting a lack of effect on NF-κB regulation. Monocyte apoptosis was examined after culture in vitro in the absence of 2 important survival factors: adherence and serum. As shown in Figure 5D, monocytes cultured in suspension in the absence of serum show a progressive decrease in the percentage of viable cells over 72 hours. The decline in the number of viable cells was accompanied by an increase in the proportion of cells that show evidence of loss of membrane permeability by failure to exclude propidium iodide (necrosis). Comparison of the proportion of viable or necrotic cells revealed a minor antiapoptotic effect for binding of unstimulated platelets to monocytes. Interestingly, monocytes coincubated with TRAP-activated platelets displayed decreased necrosis rate at all the time points analyzed compared to the corresponding control (monocytes incubated with TRAP but without platelets). Collectively, all these findings clearly indicate that in the absence of overt stimulation, platelet adhesion on monocytes had little impact on monocyte functional responses and behavior.

Discussion

Platelet-leukocyte interactions have been reported to accelerate restenosis[21] and conversely, disruption of such interactions may be beneficial in animal models of vascular injury.[22] However, whether platelet-monocyte interactions contribute to the development of cardiovascular disease is unknown. We have measured platelet-leukocyte complexes in peripheral blood from normal donors, providing the first analysis of platelet binding to the $CD14^{low}$/HLA-DR^{high} monocyte subset that is present at increased levels in the circulation during inflammatory situations.[23] Platelet binding to $CD14^{low}$/HLA-DR^{high} and $CD14^{high}$ subsets shows similar divalent cation dependency and susceptibility to blockade with P-selectin and PSGL-1 mAb. Although our data show that platelet binding to $CD14^{low}$/HLA-DR^{high} monocytes is statistically lower ($P<0.01$) than for the $CD14^{high}$ monocyte population, further studies would be required to define the biological significance of the observed difference.

Comparison of platelet binding to PMN and to monocyte subsets revealed that platelet adhesion to these cell types is predominantly mediated by P-selectin–PSGL-1 interactions. PSGL-1 blockade inhibits the majority of platelet binding to monocytes in whole blood samples, indicating that P-selectin/PSGL-1–mediated adhesion occurs physiologically. Because a very low percentage (<1%) of circulating platelets express detectable levels of P-selectin by flow cytometry, monocytes may selectively bind the P-selectin–expressing platelets present in the vasculature. Platelet-monocyte binding appears to be more stable than platelet-PMN binding, being resistant to disruption by shear stress associated with repeated vortexing. Although the $\alpha_M\beta_2$ integrin has been reported to mediate platelet–leukocyte interactions,[24] the sensitivity of platelet–monocyte interactions to the specific calcium chelator EGTA[17] suggests that β_2 integrins are unlikely to contribute to platelet binding. We observed higher levels of expression of PSGL-1, the leukocyte counter receptor for P-selectin (Figure 1B) on monocytes, and we speculate that this confers increased stability of platelet binding to monocytes compared with PMN. Prolonged platelet–monocyte interactions may differ from transient selectin-mediated leukocyte–endothelial interactions during rolling adhesion and therefore have an impact on functional behavior. In particular, sustained inter-

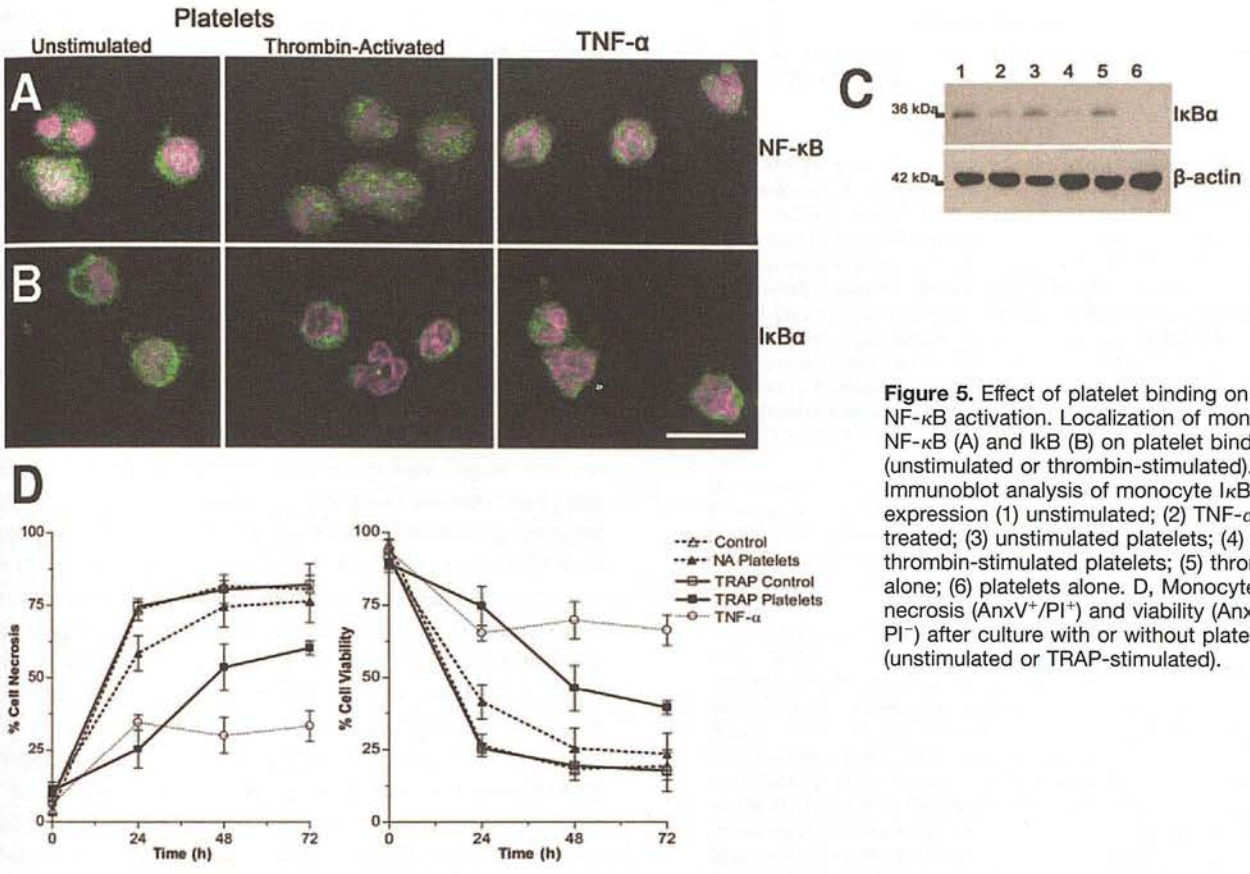

Figure 5. Effect of platelet binding on NF-κB activation. Localization of monocyte NF-κB (A) and IkB (B) on platelet binding (unstimulated or thrombin-stimulated). C, Immunoblot analysis of monocyte IκB expression (1) unstimulated; (2) TNF-α–treated; (3) unstimulated platelets; (4) thrombin-stimulated platelets; (5) thrombin alone; (6) platelets alone. D, Monocyte necrosis ($AnxV^+/PI^+$) and viability ($AnxV^-/PI^-$) after culture with or without platelets (unstimulated or TRAP-stimulated).

actions may have the potential to engage and cross-link PSGL-1 and initiate intracellular signaling pathways including phosphorylation of Syk and association of ezrin with ITAM motifs.[12,25]

Several studies have investigated the functional consequences of interactions between thrombin-activated platelets and monocytes, demonstrating production of chemokines and cytokines,[26,27] tissue factor,[28] and proteases.[29] However, many of these studies use prolonged coincubation times (18 hours), during which time additional changes in platelet or monocyte activation states may occur, for example as a result of the formation of large platelet-monocyte aggregates. Furthermore, platelet-free monocyte preparations in which platelet binding via either selectin- or integrin-dependent pathways is disrupted by divalent cation chelation (EDTA or citrate anticoagulation together with EDTA washing) were used as controls for monocytes with bound platelets. These platelet-free monocytes fail to show activation of monocyte production of chemokines, cytokines, proteases, or transcription factors,[26–29] implying that platelet binding to monocytes in the circulation fails to cause monocyte activation or alternatively that platelet-induced activation is readily reversed. Moreover, the use of P-selectin immobilized on tissue culture plates as a surrogate for PSGL-1 ligation may be very different from P-selectin binding in the context of an intact platelet. In support of this suggestion, we have recently reported that presentation of ligand on different sized latex microspheres may influence signaling pathways engaged within leukocytes. Presentation of β_2 integrin ligands on particles considerably larger than a platelet (>3 μm in diameter) was required for activation of neutrophil effector functions.[30] Finally, a recent elegant study suggests that both adhesion and cytokine signaling in combination are required to induce expression of COX-2 mRNA production and stabilization in monocytes,[31] raising the possibility that adhesive signals alone are insufficient to cause full activation of monocyte transcriptional activity. Thus, the assumption that binding of platelets to monocytes in the circulation is inevitably associated with proinflammatory consequences may be incorrect.

Our flow cytometric analyses in whole blood samples fail to reveal a difference between monocytes with and without bound unstimulated platelets in terms of monocyte surface expression of the activation-regulated molecules CD11b and CD62L, in contrast to findings reported by others.[25] However, when platelet-free monocytes are isolated either via counterflow centrifugal elutriation in divalent cation-free conditions[32] or after platelet detachment with EDTA, a distinct population of monocytes in terms of CD11b or CD62L expression corresponding to those having formerly bound platelets is not observed.

Engagement of PSGL-1 after antibody cross-linking has the potential to influence integrin-mediated adhesion and subsequent transmigration. Somewhat contrary to our expectations, we found that monocyte transmigration in response to the chemoattractant C5a was similar in the presence or absence of bound unstimulated platelets. As an additional intracellular marker for cell activation, we examined whether

unstimulated platelet binding influenced NF-κB distribution and potentially the expression of proinflammatory genes. In contrast to the observed translocation of NF-κB to the nucleus in monocytes after coincubation with thrombin-activated platelets, unstimulated platelets did not induce NF-κB redistribution. Similarly, analysis of IκBα expression by both immunofluorescence microscopy and immunoblotting revealed no differences in IκBα expression in monocytes with or without bound unstimulated platelets, whereas binding of thrombin-activated platelets caused rapid IκBα degradation. Consistent with these findings, the expression of NFκB-regulated proinflammatory cytokines, like IL-1β and TNF-α, was significantly upregulated in monocytes coincubated with thrombin-activated platelets, whereas no difference was observed between monocytes with or without bound unstimulated platelets. In this respect, binding of unstimulated platelets also failed to affect the induction of monocyte apoptosis in response to serum deprivation and suspension culture. In contrast, addition of TRAP-activated platelets had an antiapoptotic effect on monocytes, as evidenced by decreased numbers of apoptotic monocytes at all time points examined over 72 hours. Collectively, these findings clearly indicate that in the absence of overt platelet activation, platelet binding to monocytes fails to influence NF-κB activity.

In summary, we report that although platelet–monocyte and platelet–PMN interactions are mediated by PSGL-1 and P-selectin, more prolonged and stable binding to monocytes was observed. We could find no major difference in the binding of platelets to monocyte subsets, and examination of a number of different monocyte functional attributes suggested that binding of unstimulated platelets did not affect receptor expression, cytokine production, NF-κB activation, chemotactic responses, or apoptosis. In contrast, binding of activated platelets does trigger proinflammatory responses in monocytes. One possibility is that high levels of P-selectin on the surface of activated platelets or binding of multiple platelets per monocyte is required to trigger monocyte activation via PSGL-1. In addition, release of a range of proinflammatory cytokines, including IL-1β, IL-6, and IL-12 after platelet activation (data not shown), might provide additional signals that lower the threshold for monocyte responsiveness. In this context, based on the reported increase in thrombin-mediated platelet activation in patients with ACS,[33,34] it seems possible that under these conditions, monocyte-platelet interactions lead to enhanced proinflammatory responses which in turn exacerbate vascular inflammation. In contrast, in the absence of platelet activation, PSGL-1–mediated platelet adhesion to circulating monocytes represent a physiological process with little impact on cell physiology, and the assumption that such interactions in peripheral blood have proinflammatory consequences should be carefully reconsidered.

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Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin

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Apoptosis is a noninflammatory, programmed form of cell death. One mechanism underlying the non-phlogistic nature of the apoptosis program is the swift phagocytosis of the dying cells. How apoptotic cells attract mononuclear phagocytes and not granulocytes, the professional phagocytes that accumulate at sites of inflammation, has not been determined. Here, we show that apoptotic human cell lines of diverse lineages synthesize and secrete lactoferrin, a pleiotropic glycoprotein with known antiinflammatory properties. We further demonstrated that lactoferrin selectively inhibited migration of granulocytes but not mononuclear phagocytes, both in vitro and in vivo. Finally, we were able to attribute this antiinflammatory function of lactoferrin to its effects on granulocyte signaling pathways that regulate cell adhesion and motility. Together, our results identify lactoferrin as an antiinflammatory component of the apoptosis milieu and define what we believe to be a novel antiinflammatory property of lactoferrin: the ability to function as a negative regulator of granulocyte migration.

Introduction

Apoptosis is a programmed, physiological form of cell death that, in inflammatory terms, is quiet: apoptotic cells are rapidly phagocytosed by their neighbors or by mononuclear phagocytes that are attracted by chemotactic factors such as lysophosphatidylcholine (1) and fractalkine (2) released by apoptotic cells. The rapid engulfment of apoptotic cells militates against the potential tissue-injuring and proinflammatory features of dead cells as exemplified by necrosis (3–6). Indeed, failed or delayed clearance of apoptotic cells can have detrimental inflammatory consequences, including the development of autoimmune pathologies (7–9). The mechanisms underlying the non- or antiinflammatory nature of the apoptosis program are not understood in detail. Antiinflammatory mediators such as TGF-β1 and IL-10 are known to be produced at sites of apoptosis, both directly by apoptotic cells themselves and indirectly through interaction of apoptotic cells with phagocytes (10, 11). Apoptotic cells selectively attract mononuclear phagocytes (1, 12), but, curiously, the other class of professional phagocytes, granulocytes or polymorphonuclear phagocytes, do not migrate toward apoptotic cells in vitro (12) and do not normally engage in apoptotic cell engulfment. The absence of granulocytes from sites of homeostatic and developmental apoptosis suggests that apoptotic cells have the potential to selectively regulate the recruitment of mononuclear leukocytes.

In marked contrast to sites of apoptosis, sites of acute infection are characterized by the presence of granulocytes, most commonly neutrophils, as a means to protect the host by engulfing, killing, and digesting invading infectious agents. Neutrophils, as a first line of immune defense, are rapidly recruited to the site of infection in response to a variety of inflammatory stimuli, including chemokines, cytokines, leukotrienes, and bacterial components such as LPS and *N*-formylated peptides (13, 14). Comparative studies on the slime mold *Dictyostelium discoideum* have shown that in response to chemoattractants, neutrophils orient themselves and migrate in an "ameboid motion" by anterior pseudopod extension accompanied by simultaneous posterior contraction and retraction. Such polarized morphology is characterized by the formation of a lamellipodium at the leading edge and a uropod at the trailing edge of the neutrophil (15–18). This process is tightly controlled not only to ensure the efficient migration of neutrophils to inflammatory sites, but also to prevent their aberrant infiltration and consequent tissue-damaging activities. Detrimental effects of neutrophils — caused, for example, by release of their proteolytic enzymes — contribute to many pathological inflammatory conditions, ranging from vasculitis and ischemia/reperfusion injury to glomerulonephritis, rheumatoid arthritis, and acute graft rejection (19). Therefore, at inflammatory sites, negative signals exist that prevent neutrophil recruitment, dampen neutrophil responsiveness, and counterbalance or terminate the inflammatory response. This antiinflammatory program is characterized by cessation of neutrophil infiltration, as arachidonic acid–derived prostaglandins and leukotrienes are switched to lipoxins, resolvins, and protectins (20, 21). Resolution of inflammation is ultimately achieved through neutrophil apoptosis, and apoptotic neutrophils are subsequently phagocytosed by macrophages, a process that leads to the release not only of antiinflammatory cytokines but also of such antiinflammatory and proresolving lipid mediators as lipoxin A4, resolvin E1, and protectin D1 (22–25).

Given (a) the production of negative signaling molecules at inflammatory sites to limit neutrophil recruitment and function and (b) failure of neutrophil migration to sites of apoptosis, we sought to determine whether apoptotic cells actively produce negative regulators of neutrophil chemotaxis. We postulated that the production by apoptotic cells of factors that inhibit neutrophil migration contribute to the non-phlogistic nature of the apoptosis program. Here, we present evidence that apoptotic cells actively inhibit neutrophil migration through the production of

Conflict of interest: C.D. Gregory and J.D. Pound are founders of the company, ImmunoSolv Ltd., that supplied 2 of the monoclonal anti-lactoferrin antibodies used in Supplemental Figure 1.

Nonstandard abbreviations used: BL, Burkitt lymphoma; $[Ca^{2+}]_i$, intracellular calcium concentration; fMLP, formyl-methionyl-leucyl-phenylalanine; LTB_4, leukotriene B_4.

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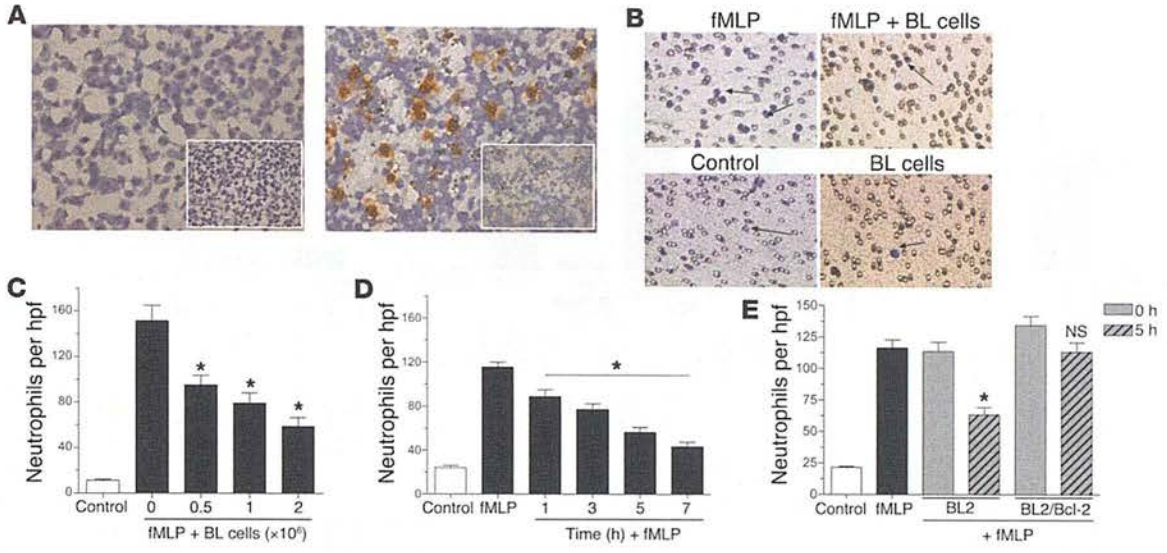

Figure 1

Apoptotic cells release factor(s) that inhibit neutrophil migration. (**A**) Immunohistochemical analysis of neutrophils in BL (left) and spleen (positive control; right) sections. Inset images represent isotype control. (**B**) Representative images of stained Transwell filters. (**C**) Neutrophil chemotaxis toward increasing concentrations of BL cells was assessed in the presence of fMLP (100 nM). $n = 3$; $^*P < 0.05$ vs. time 0. (**D**) BL cell–conditioned media obtained at the indicated time points were used to analyze fMLP-induced neutrophil chemotaxis. $n = 3$; $^*P < 0.05$ vs. fMLP. (**E**) Neutrophil chemotaxis toward fMLP was analyzed in the presence of control or Bcl-2–transfected BL2 cells obtained following a 0- and 5-hour incubation at 37°C. $n = 3$; $^*P < 0.05$ vs. BL2 0 h; NS vs. BL2/Bcl-2 0 h. Apoptosis levels were assessed by flow cytometry following staining with annexin V/propidium iodide. Error bars indicate SEM. Original magnification; ×400 (**A**; **A**, insets; **B**). hpf, high-power field.

lactoferrin, an 80 kDa antiinflammatory glycoprotein that specifically inhibits chemotaxis of neutrophils but not mononuclear phagocytes. Lactoferrin was found to be synthesized de novo following triggering of apoptosis and released by apoptotic cells of diverse lineages. Lactoferrin impaired neutrophil activation and prevented mobilization of the cells by inhibiting migratory polarization of the cell body. These results demonstrate that the non-phlogistic constitution of apoptotic cells includes an anti-inflammatory molecule, lactoferrin, that we show, for the first time to our knowledge, has potent negative regulatory effects on neutrophil migration. These findings provide a rationale for the absence of neutrophils from apoptotic sites and have important implications for understanding the mechanisms involved in the resolution phase of inflammation.

Results

Apoptotic cells actively produce factor(s) that inhibit neutrophil chemotaxis. To address whether apoptotic cells influence migratory activity of neutrophils, we carried out a series of in vitro Boyden-type chemotaxis assays to investigate neutrophil migration toward Burkitt lymphoma (BL) cells. We initially employed BL as a model tissue as these tumor cell populations display high levels of apoptosis, a property that is retained constitutively in the tumor-derived cell lines. As at all sites of apoptosis, there is marked infiltration of macrophages that engulf the apoptotic cells, giving rise to the typical "starry sky" histological appearance of this tumor. As shown in Figure 1A (left), while macrophages were abundant in histological sections of BL, neutrophils were absent. We assessed the effects of BL cells on the migratory activity of neutrophils in vitro by adding neutrophils to the top compartment of a Transwell filter and inducing them to migrate toward the lower chamber containing BL cells in the presence of the powerful neutrophil chemoattractant formyl-methionyl-leucyl-phenylalanine (fMLP) (Figure 1B). As shown in Figure 1C, neutrophil migration was significantly inhibited in BL cells in a concentration-dependent manner. We observed a similar effect irrespective of the chemoattractant used (inhibition of neutrophil migration induced by C5a, IL-8, and leukotriene B_4 [LTB_4]; data not shown, but see below). We carried out subsequent chemotaxis assays using BL-conditioned medium obtained over a 7-hour time course and found that BL cells actively released an inhibitory factor(s) (Figure 1D). The release of the inhibitory factor appeared to be linked to the levels of apoptosis in the BL cell populations, since the inhibitory activity was significantly lower in further chemotaxis assays using BL-conditioned medium derived from cells overexpressing the apoptosis inhibitor Bcl-2, as compared with that of parental cells (Figure 1E).

Biochemical characterization of neutrophil migration–inhibitory factor(s). In an attempt to gain further insight into the biochemical nature of the factor(s) that BL cells secrete in order to exclude neutrophils from their environment, we initially estimated the molecular weight range of the inhibitory factor(s) by using filters with molecular weight cutoff points of approximately 3, 10, 30, 50, and 100 kDa. BL-conditioned media obtained after 24-hour incubation were fractionated, and each fraction was examined in vitro using the neutrophil chemotaxis assay described above. The results revealed that fractions containing molecules of less than 50 kDa failed to display any inhibitory effect on neutrophil migration (Figure 2A). However, the use of 100 kDa filters revealed that both fractions (>100 kDa and <100 kDa) displayed an inhibitory effect on neutrophil migration, indicating that at least 1 factor has a molecular weight that ranges between 50 and 100 kDa. The presence of inhibitory activity in the filtrate of the 100 kDa

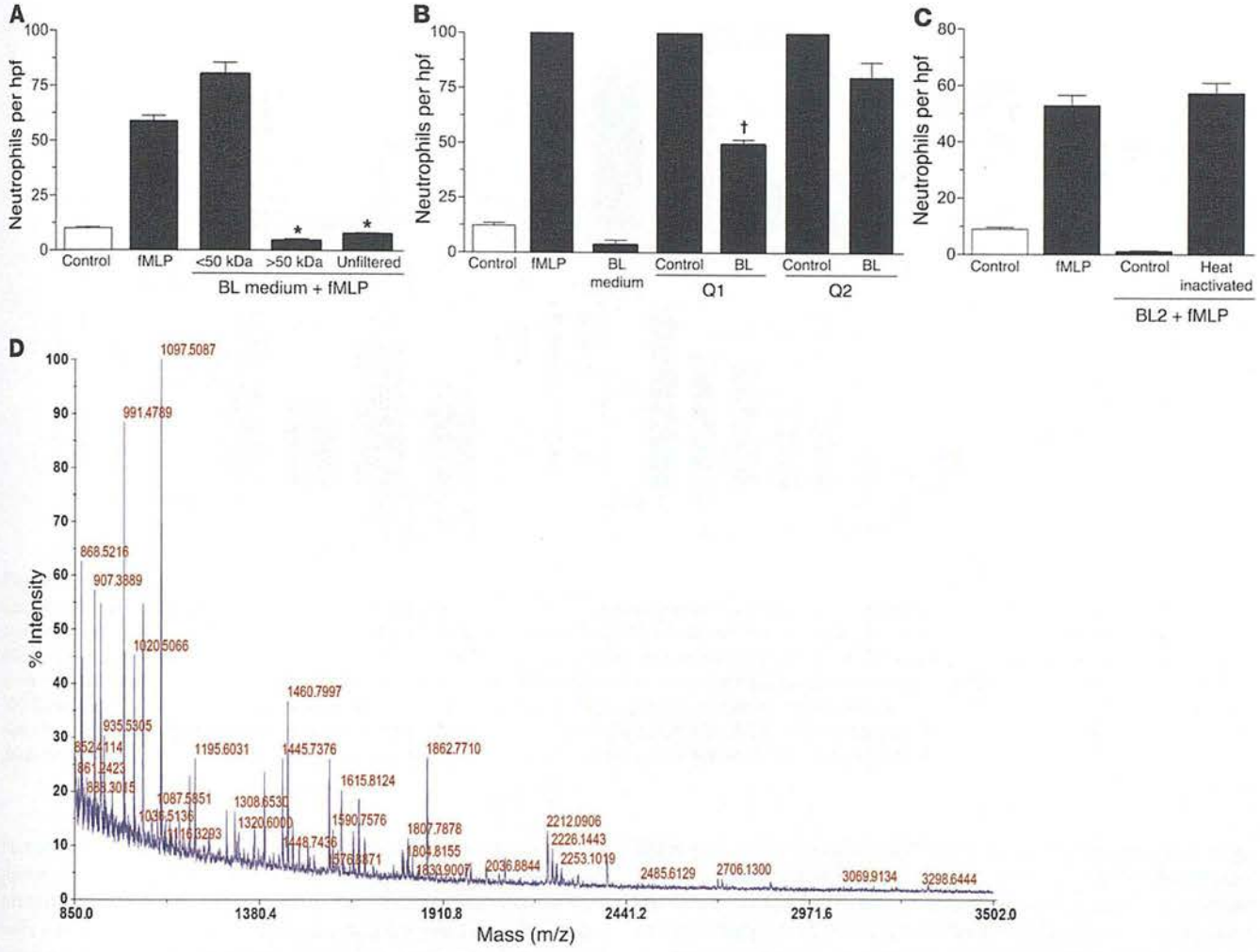

Figure 2

Biochemical characterization of the inhibitory factor. Conditioned media from BL2 cells cultured for 24 hours were size fractionated using filters with 50 kDa (**A**) molecular weight cutoff sizes. Unfiltered medium was included as control. $^{*}P < 0.001$ compared with the corresponding positive control. Error bars indicate SEM. Ion exchange analysis included the use of Q Sepharose beads (positively charged) in order to distinguish positively and negatively charged molecules in the <100 kDa fraction (**B**) of the BL medium. Unbound molecules (Q1 fraction) were collected, whereas bound molecules were eluted from the beads (Q2 fraction). Neutrophil migration toward these fractions in the presence of fMLP (100 nM) was assessed. Q1 and Q2 fractions (unbound and eluant fraction) of serum-free medium (no BL) were included as control. $^{\dagger}P < 0.05$ compared with the corresponding control. Error bars indicate SEM. (**C**) Chemotaxis assay of neutrophils toward BL-conditioned medium that was heat inactivated (90°C for 10 minutes). (**D**) MALDI-TOF mass spectrum for the tryptic digest of the peptide band identified as lactoferrin.

cutoff membrane is likely to result from (a) imprecise molecular weight cutoff of molecules in the 50–100 kDa range, (b) complex formation through multimerization of the 50–100 kDa factor or through interaction with other molecules, or (c) the existence of a distinct inhibitory activity of greater than 100 kDa. It should be noted that the selected isolation approach is skewed in favor of proteins and that additional low-molecular-weight (for example, lipid) mediators of neutrophil migration inhibition would not be identified by these procedures.

To investigate further the biochemical properties of the retentate and filtrate of the 100 kDa cutoff membrane, we first determined the charge (pI value) of the migration-inhibitory activity by means of an ion exchange analysis of BL-conditioned media. Using positively charged ion exchange beads (Q Sepharose), the 100 kDa membrane retentate and filtrate were separated into positively charged (Q-supernatant) and negatively charged (Q-eluant) fractions. As shown in Figure 2B, the filtrate of the 100 kDa membrane (<100 kDa fraction) contained migration-inhibitory activity in both the supernatant (positive charge) and the eluant (negative charge) of the Q beads. By contrast, analyses of the retentate (>100 kDa fraction) revealed that only the negatively charged eluant displayed significant activity in inhibiting neutrophil migration. These results indicate that at least 2 moieties with neutrophil migration–inhibitory activity were present in BL cell–conditioned medium: one of 50–100 kDa with positive pI and a second of 100 kDa or more and negatively charged.

We subsequently determined whether the neutrophil migration-inhibitory activity was heat labile. As shown in Figure 2C, heat inactivation completely abrogated all chemotaxis inhibitory activity in BL cell–conditioned medium, suggesting that the inhibitory

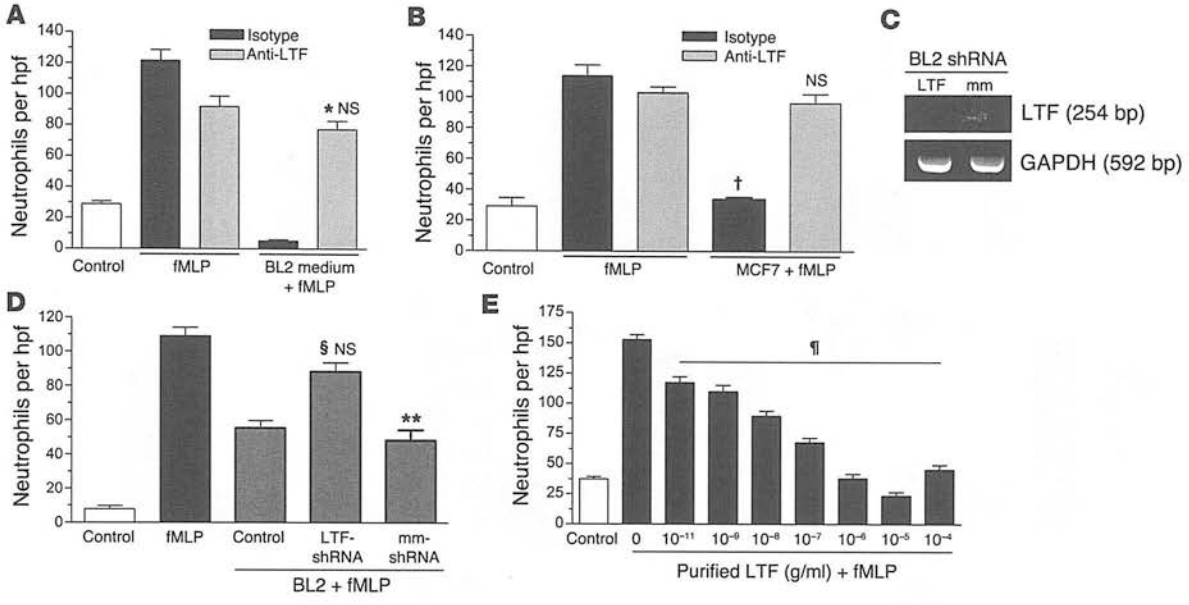

Figure 3

Lactoferrin specifically inhibits neutrophil chemotaxis. Neutrophil chemotaxis in the presence of human anti-lactoferrin (anti-LTF) polyclonal antibody (gray) or isotype control (black) using conditioned media from BL (**A**) and MCF7 (**B**) cells (**A**: $n = 3$, $^{*}P < 0.05$ vs. isotype control, NS vs. fMLP anti-lactoferrin control; **B**; $n = 3$, $^{\dagger}P < 0.001$ vs. fMLP/isotype; NS vs. fMLP/anti-LTF. (**C**) RT-PCR analysis to assess lactoferrin expression in BL cells stably expressing LTF shRNA (LTF) cells and mock-transfected (mm) cells induced to become apoptotic (1 μM staurosporine; 37°C). (**D**) Chemotaxis assay to determine neutrophil migration toward supernatants obtained from control, LTF shRNA, and mock-transfected BL cells ($n = 5$; $^{\S}P < 0.05$ compared with mm shRNA control; $^{**}P < 0.05$ compared with fMLP; NS compared with fMLP control). (**E**) Dose-response analysis of purified human lactoferrin. $n = 3$; $^{\P}P < 0.05$ vs. 0 g/ml purified LTF + fMLP. Error bars indicate SEM.

factor(s) were most likely protein in nature. We then analyzed the proteins released from BL cells in viable and apoptotic states by protein fingerprinting. Polypeptide bands of greater than 50 kDa were excised from a 10% SDS polyacrylamide gel. Tryptic peptides were gel extracted, and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometric analysis was carried out (Figure 2D). Also, given the crude biochemical characteristics described above, we undertook a candidate approach based on the proteins released from apoptotic BL cells. We identified the factor released by BL cells that prevented neutrophil chemotaxis as lactoferrin.

Lactoferrin specifically inhibits neutrophil chemotaxis toward a range of chemoattractants. Lactoferrin is a glycoprotein of approximately 75–80 kDa that belongs to the transferrin family of proteins due to its iron-binding properties. It is a well-characterized component of neutrophil secondary granules, lacrimal fluid, colostrum, saliva, and mucosal secretions, in which it confers antibacterial activity. We observed that addition of anti-lactoferrin antibody to BL-conditioned medium neutralized its neutrophil migration–inhibitory activity using either polyclonal or monoclonal antibodies (Figure 3A and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI36226DS1). Similar results were observed with supernatants obtained from the mammary carcinoma line MCF-7, indicating that the neutrophil migration–inhibitory activity is not restricted to BL cell–derived lactoferrin (Figure 3B). Furthermore, lactoferrin purified from human milk displayed dose-dependent inhibitory activity toward neutrophil migration in response to fMLP (Figure 3E) and also inhibited migration toward C5a, IL-8, and LTB_4 to similar levels (Figure 4A). The neutrophil migration–inhibitory effect was also displayed by lactoferrin purified from human neutrophils (Figure 4D). It should be noted that both types of purified lactoferrin used in this study were free of endotoxin contamination, and the observed inhibitory effect did not appear to be due to any lactoferrin-associated molecules such as LPS. Furthermore, inhibition of lactoferrin expression by shRNA in BL cells provides additional support for the specificity of the observed lactoferrin effect. Thus, we found that neutrophil chemotaxis toward supernatants obtained from BL2 cells transfected with shRNA vectors targeted against lactoferrin was higher compared with supernatants from BL control or mock-transfected cells (Figures 3, C and D). Lactoferrin exerted no toxic effects on neutrophils, as assessed by annexin V/propidium iodide staining of control and lactoferrin-treated neutrophils (>98% cell viability). These results suggested that lactoferrin binds to neutrophils and inhibits their ability to undergo chemotaxis. To exclude the possibility that the observed inhibitory activity of lactoferrin was due instead to its ability to bind and functionally neutralize the chemoattractants, additional chemotaxis assays were performed using chemoattractants (fMLP, C5a, IL-8) that were preabsorbed with lactoferrin. To achieve this, we preincubated chemoattractants with lactoferrin. Subsequently, anti-lactoferrin antibody was used to remove the lactoferrin with the aid of magnetic beads. As shown in Figure 4B, no difference in neutrophil chemotactic activity was observed between the control and lactoferrin-absorbed chemoattractants, which excludes the possibility that lactoferrin binds to, and alters the activity of, the chemoattractants. Further supporting our conclusion that lactoferrin exerts its inhibitory effects through binding to neutrophils, we also observed that purified lactoferrin can directly associate with neutrophils (Figure 4C). In addition, Scatchard binding

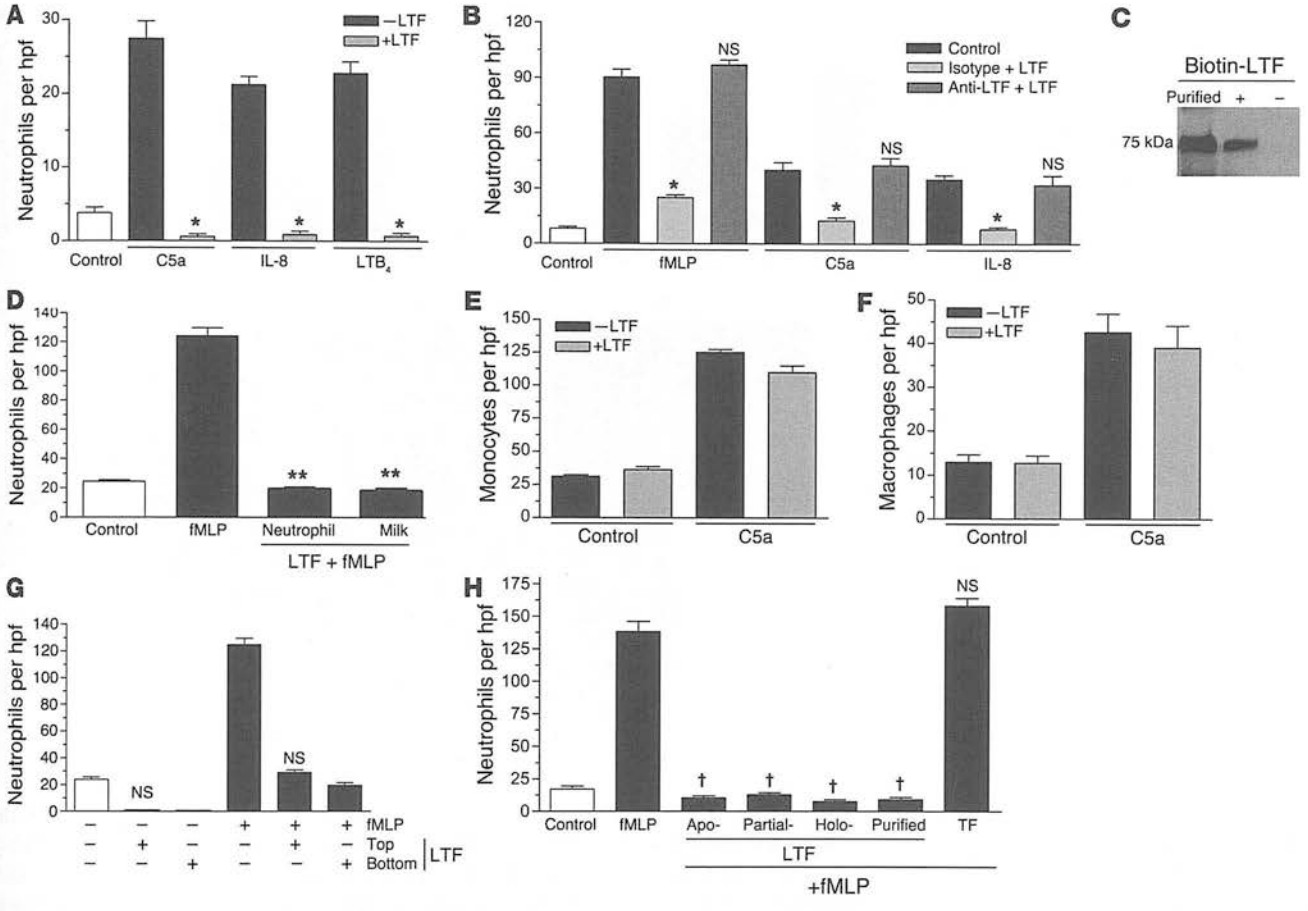

Figure 4

Neutrophil chemotaxis toward lactoferrin is irrespective of the chemoattractant used and its iron saturation status. (**A**) Neutrophil chemotaxis toward different chemoattractants. $n = 3$; $^{*}P < 0.05$. (**B**) Neutrophil chemotaxis toward chemoattractants (control) or chemoattractants that were incubated with lactoferrin (10 μg/ml) followed by the addition of isotype or anti-lactoferrin monoclonal antibody (10 μg/ml). Antibodies were removed using magnetic IgG beads. $n = 3$; $^{*}P < 0.05$, NS compared with chemoattractant control. (**C**) Immunoblot analysis of lysates of neutrophils incubated with or without biotinylated lactoferrin (10 μg/ml) at 37°C for 1 hour. (**D**) Neutrophil chemotaxis toward lactoferrin (10 μg/ml) purified from human neutrophils or human milk. $^{**}P < 0.001$ vs. fMLP. C5a-induced monocyte (**E**) or macrophage (**F**) chemotaxis. (**G**) Neutrophil migration in the presence of lactoferrin (10 μg/ml) in the top or bottom compartment of the Transwell insert ($n = 3$; NS vs. corresponding +LTF controls). (**H**) Chemotaxis assay to determine neutrophil migration toward purified recombinant iron-depleted (Apo-), partially iron-saturated, and fully iron-saturated (Holo-) recombinant lactoferrin (10 μg/ml). Milk-purified lactoferrin and partially iron-saturated transferrin (TF; 10 μg/ml) were added as control. $n = 4$; $^{\dagger}P < 0.001$ compared with fMLP control. Error bars indicate SEM.

analysis of ^{125}I-labeled apolactoferrin indicated, in accordance with earlier findings (26, 27), that lactoferrin bound to neutrophils via 2 classes of receptor that differ in affinity and number of binding sites per cell. We determined the higher-affinity receptors to be expressed at a density of 9,100 ± 2,500 binding sites per cell, with an affinity of 350 ± 65 nM, and the lower-affinity receptors to be expressed at a density of $2.5 \times 10^6 \pm 0.7 \times 10^6$ per cell, with an affinity of 20 ± 10 μM (Supplemental Figure 2).

To determine whether the migration-inhibitory effects of lactoferrin were specific to neutrophils among professional phagocytes, we analyzed its effects on monocyte and macrophage migration in vitro. As shown in Figure 4, E and F, C5a-induced chemotaxis of mononuclear phagocytes was unimpaired by lactoferrin. We further assessed whether lactoferrin acted by inhibiting neutrophil migration or promoting neutrophil repulsion. In chemotaxis assays, in which we added lactoferrin to the upper chamber along with neutrophils, we observed inhibition of neutrophil migration toward fMLP and control medium (Figure 4G), suggesting that lactoferrin exerts a direct effect on neutrophils by inhibiting their migratory ability and not by forcing them to migrate in all directions away from the chemoattractant.

Neutrophil migration–inhibitory effects of lactoferrin are not related to its iron-binding properties and are demonstrable in vitro and in vivo. Iron and iron-associated molecules have been previously shown to play an important role in many immunomodulatory functions. Indeed, suppression of IL-1 release by monocytes is observed by purified iron-saturated lactoferrin, whereas an inhibition of GM colony-stimulating activity production by monocytes and macrophages correlated with the iron saturation status of lactoferrin (28–30). Therefore, we further examined whether differences in the iron saturation profile of lactoferrin affect its ability to inhibit neutrophil migration. Chemotaxis assays to determine neutrophil migration toward iron-depleted (apo-lactoferrin), partially iron-saturated, or fully iron-saturated (holo-lactoferrin) lactofer-

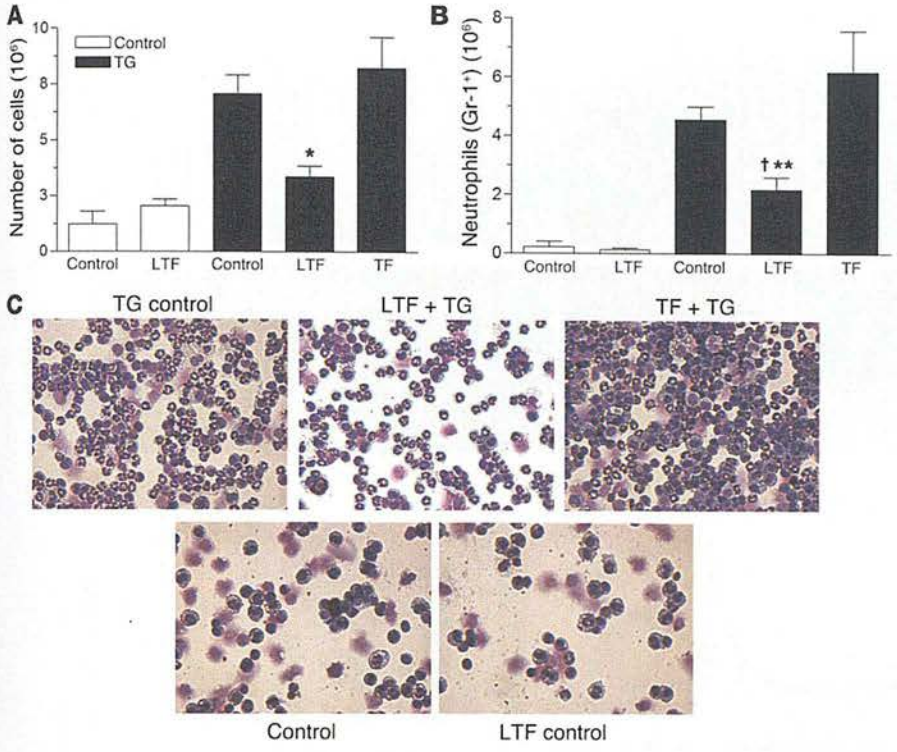

Figure 5
Lactoferrin inhibits neutrophil migration in vivo. Total cell (**A**) or neutrophil number (Gr-1$^+$; **B**) obtained from peritoneal lavage. *$P < 0.05$ vs. transferrin; †$P < 0.05$ vs. thioglycollate (TG) control, **$P < 0.01$ vs. transferrin control. Error bars indicate SEM. (**C**) Characteristic cytospin images. Original magnification, ×400, top; ×200, bottom.

rin revealed that the level of iron saturation was not responsible for the observed inhibition in neutrophil migration (Figure 4H). Also, as lactoferrin belongs to the transferrin family of proteins sharing 74% sequence homology with transferrin (both of them are 80 kDa cationic iron-binding glycoproteins), we reasoned that, if the underlying neutrophil migration–inhibitory mechanism of lactoferrin was rooted in its ability to chelate iron, transferrin might show similar effects on neutrophil migration. To explore this possibility, we performed chemotaxis assays in which neutrophils were induced to migrate toward fMLP in the presence of partially iron-saturated transferrin. Our results showed that transferrin, unlike lactoferrin, had no effect on neutrophil chemotaxis, further supporting the conclusion that the observed neutrophil migration–inhibitory effect is lactoferrin specific and is unlikely to require iron-chelating activity (Figure 4H).

Having established the inhibitory effects of lactoferrin on neutrophil chemotaxis in vitro, we then used a murine peritonitis model to assess the effect of lactoferrin on leukocyte recruitment in vivo. Lactoferrin and transferrin were tested for their ability to affect thioglycollate-induced leukocyte recruitment to the peritoneal cavity. As shown in Figure 5, A and B, thioglycollate caused rapid recruitment of leukocytes compared with vehicle alone, and the recruited leukocytes were predominantly neutrophils (88%). In the presence of lactoferrin, the total number of neutrophils recruited to the peritoneal cavity was reduced by 52% compared with control, whereas transferrin had no effect. Lactoferrin reduced specifically the proportion and number of neutrophils migrating into the cavity but did not affect recruitment of other types of leukocytes in response to thioglycollate (Figure 5C). These results demonstrate that, similar to its effect on neutrophil chemoattraction in vitro, lactoferrin is a potent inhibitor of neutrophil migration in vivo.

Impairment of neutrophil activation profile by lactoferrin. Neutrophil migration involves activation, adhesion, and extravasation processes that are accompanied by gross changes in cell morphology: whereas nonactivated neutrophils are rounded, activated neutrophils acquire a polarized morphology with spreading and adhesion to the available substratum (15). In order to initially assess the effects of lactoferrin on neutrophil activation, we performed time-lapse video microscopy of neutrophils and recorded directly their activation morphology, cell spreading, and locomotion. During a 1-hour time course, lactoferrin-pretreated neutrophil populations stimulated with fMLP displayed a greater proportion of nonadherent cells as well as cells presenting a rounded, nonactivated morphology as compared with neutrophils treated with fMLP alone (Figure 6, A and B). These quantitative differences between lactoferrin-treated and untreated neutrophils stimulated with fMLP were reflected in the locomotion of the cells around the substratum, with lactoferrin-treated cells displaying markedly reduced movement.

Changes in cell morphology following stimulation with fMLP or other neutrophil agonists are characterized by a rapid increase in intracellular cytoplasmic calcium levels through mobilization of calcium from ER stores and activation of calcium influx channels of the plasma membrane, mediated by the inositol triphosphate (IP_3) and diacylglycerol/PLC (DAG/PLC) pathways (31–33). In order to determine whether the observed cell shape alterations following lactoferrin treatment are related to changes in intracellular calcium concentrations ($[Ca^{2+}]_i$), we measured the levels of $[Ca^{2+}]_i$ in control and lactoferrin-treated cells in response to fMLP stimulation (1 nM or 10 nM). No changes were observed in the fMLP-mediated $[Ca^{2+}]_i$ response between control and lactoferrin-treated neutrophils, suggesting that lactoferrin acts downstream or independently of the mechanisms involved in intracellular calcium flux (Figure 6C).

As lactoferrin was shown to prevent neutrophil migration, we next explored whether it could also affect the neutrophil activation state. To this end, we chose to measure the expression of 2

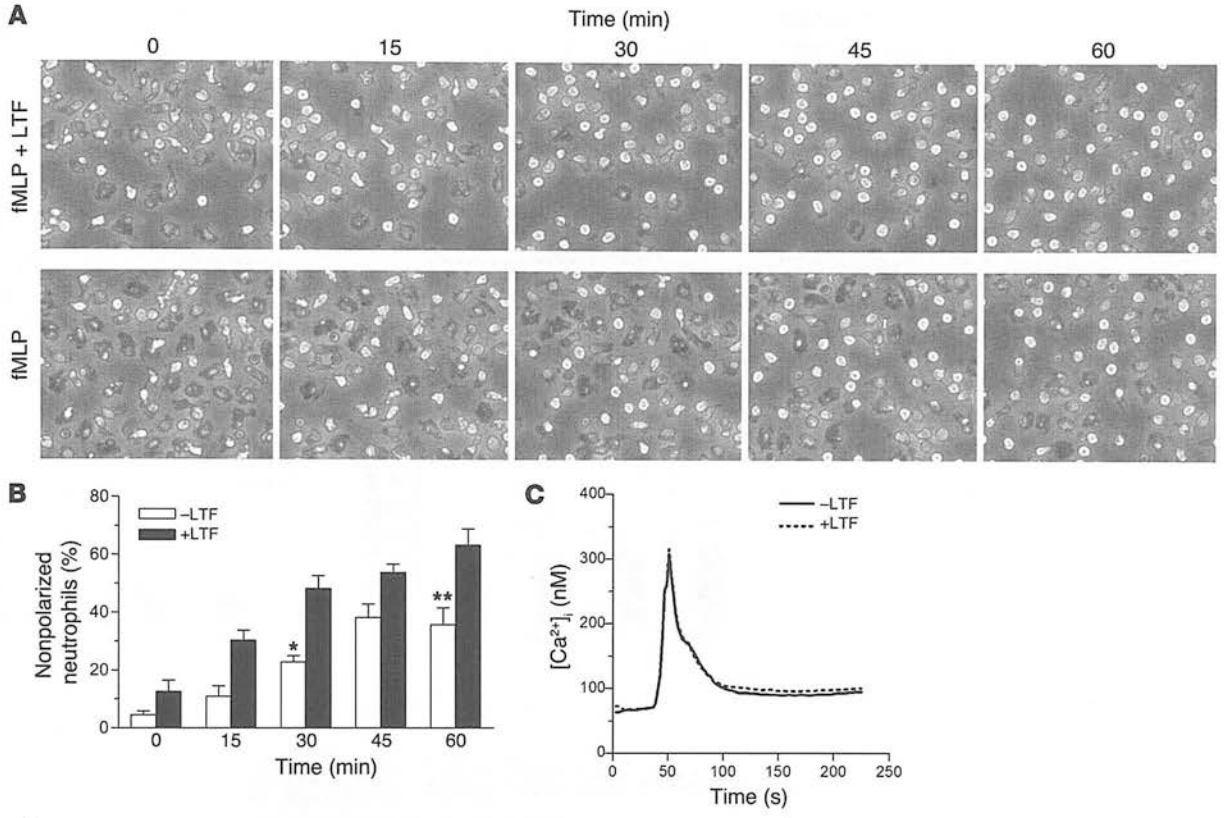

Figure 6

Effect of lactoferrin on neutrophil polarization morphology and spreading. (**A**) Time-lapse video microscopy frames of control or lactoferrin-pretreated neutrophils (10 μg/ml; 40 minutes at 37°C) stimulated with 1 μM fMLP over a 1-hour incubation time course. Original magnification, ×400. (**B**) Quantification of neutrophils (nonpolarized) counted from 5 different fields; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. corresponding +LTF control. Error bars indicate SEM. (**C**) Representative plot (of 3 independent experiments) showing measurement of $[Ca^{2+}]_i$ levels in neutrophils incubated in the presence or absence of lactoferrin (10 μg/ml; 30 minutes at 37°C) followed by stimulation with 10 nM fMLP.

known neutrophil activation–associated markers, CD62L (L-selectin) and CD11b, using 2-color flow cytometry. Upon activation, CD62L is cleaved from the neutrophil surface, whereas CD11b expression is upregulated following translocation from cytoplasmic granules to the cell membrane. Freshly isolated neutrophils were pretreated with lactoferrin and then exposed to the activation stimuli fMLP, TNF-α, and PMA. As shown in Figure 7, A–D, we found that, in lactoferrin-treated neutrophils compared with control cells, CD62L expression was significantly higher, whereas CD11b levels were lower. These effects were common to all activation stimuli used. Transferrin-treated neutrophils were also included but showed no significant differences compared with control cells. It is noteworthy that the lactoferrin effect was also evident when PMA, a specific PKC activator, was used as an agonist, indicating that lactoferrin acts downstream of PKC and not on pathways involved in PKC activation and $Ca^{2+}{}_i$ responses, such as the IP_3 and DAG/PLC pathways. This finding prompted us to investigate putative downstream targets of PKC involved in the late signaling cascades following neutrophil activation that also regulate cell motility and actin reorganization. Such cascades involve the activation of MAP family kinases (34), and we therefore examined the phosphorylation of p44/42 (ERK1 and ERK2) MAPKs. Whereas in untreated neutrophils, ERK1 and ERK2 were phosphorylated following fMLP stimulation (100 nM), lower levels of phosphorylated ERK1/2 were observed in neutrophils that had been pretreated with lactoferrin prior to stimulation with fMLP (Figure 7E). Collectively, these data suggest that lactoferrin has a clear impact on neutrophil activation, including impairment of neutrophil degranulation, inhibition of expression of β_2 integrins, and reduction of activation of intracellular kinases, with profound effects on cell migration and motility.

Induction of apoptosis upregulates lactoferrin expression and release in diverse cell types. Pursuing our initial hypothesis, which was strengthened by early observations that inhibition of neutrophil migration by BL cells appeared to be correlated with BL cell apoptosis (Figure 1E), we assessed lactoferrin expression following induction of apoptosis in a panel of cells of diverse lineages. By transcriptional analysis using RT-PCR, we found that lactoferrin was expressed, as reported previously (35), by MCF-7 mammary epithelial cells in their viable state but not by Jurkat, BL2, or A549 cells. Upon apoptosis induction, lactoferrin expression was upregulated in MCF-7 cells and expressed de novo in Jurkat, BL2, and A549 cells (Figure 8). More specifically, lactoferrin was transcribed de novo early after induction of apoptosis in A549 cells by either 100 nM etoposide or 1 μM staurosporine (Figure 8B). Levels of lactoferrin induced by etoposide were reduced in cells treated in the presence of the broad-spectrum caspase inhibitor zVAD-fmk, which prevented apoptosis induction (Figure 8C). The link between lactoferrin expression and apoptosis induction was further supported by the effects of the apoptosis inhibitor Bcl-2.

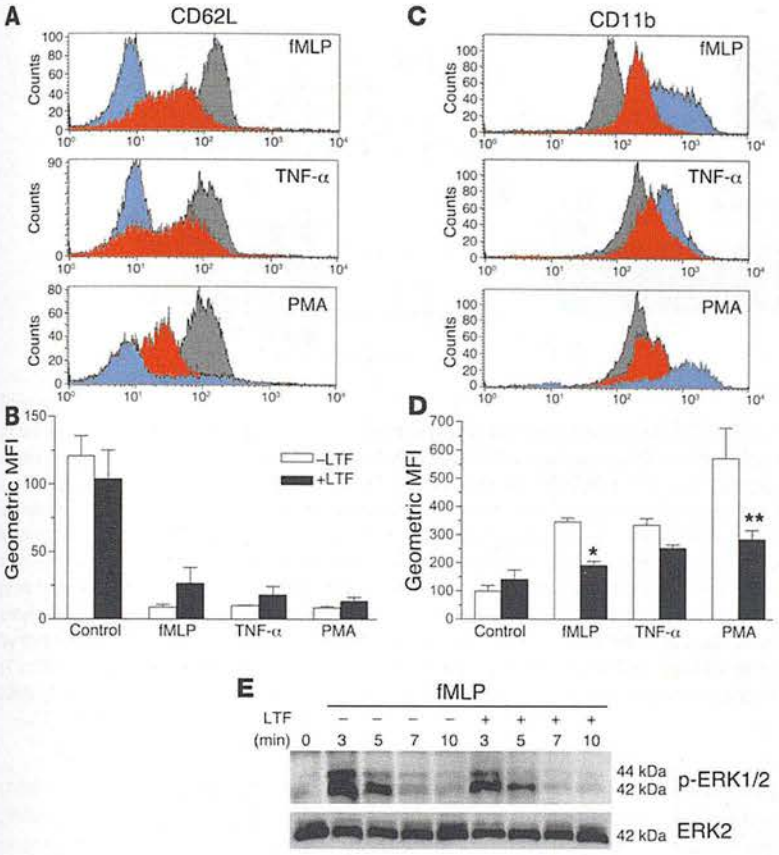

Figure 7

Effect of lactoferrin on neutrophil activation status. The expression of CD62L (**A** and **B**) and CD11b (**C** and **D**) was assessed in fMLP- (100 nM), TNF-α– (1 ng ml), or PMA-stimulated (100 nM) neutrophils (30 minutes at 37°C) that were preincubated (40 minutes at 37°C) in the presence or absence of lactoferrin (10 μg/ml). Representative flow cytometry overlays of CD62L (**A**) and CD11b (**C**) expression in control (gray) and stimulated neutrophils (lactoferrin-treated: red; untreated: blue). $n = 3$; $^*P < 0.05$, $^{**}P < 0.01$. Error bars indicate SEM. (**E**) Western blot analysis to determine levels of ERK1/2 phosphorylation. Neutrophils were incubated with lactoferrin (10 μg/ml; 40 minutes at 37°C), followed by stimulation with fMLP (100 nM) for the indicated times. Membrane was stripped and reprobed for total ERK2. Results are representative of 3 independent experiments.

BL cells expressing exogenous Bcl-2 that provided protection from apoptosis expressed lower levels of lactoferrin upon exposure to staurosporine than did their parental counterparts (Figure 8, A and D). Not only was apoptosis-related lactoferrin expression demonstrated at the transcriptional level, lactoferrin protein was also recovered from supernatants of cells undergoing apoptosis (Figure 8D). Treatment of A549 cells with brefeldin A, which interferes with intracellular transport of newly synthesized proteins, resulted in inhibition of apoptosis-induced lactoferrin release, providing further evidence for de novo synthesis and secretion of lactoferrin by cells undergoing apoptosis (Figure 8E). An analogous effect was also evident from supernatants obtained from primary lymphocytes induced to become apoptotic in the presence of 1 μM staurosporine. Finally, immunoblotting analyses and chemotaxis assays using supernatants of BL cells undergoing primary necrosis further revealed that the release of lactoferrin is not linked to necrosis but that lactoferrin is expressed and actively released from cells as a consequence of activation of their apoptosis program (Figure 8F and Supplemental Figure 3).

Discussion

Apoptosis plays a key role in regulating cell populations through programmed cell death, an intrinsically non-phlogistic process that contrasts with accidental, necrotic cell death, which has proinflammatory consequences. Defining the properties of apoptotic cells that contribute to the noninflammatory or antiinflammatory nature of the apoptosis program is critical to our understanding of this fundamental biological process. We have now identified lactoferrin as a cell-autonomous antiinflammatory mediator that is produced by cells of diverse lineages undergoing apoptosis. Furthermore, these studies demonstrate a hitherto unrecognized immunomodulatory function of lactoferrin: to inhibit directly the migration of neutrophil granulocytes. Lactoferrin is therefore a constitutive antiinflammatory component of apoptotic cells that, in addition to its known antiinflammatory properties, militates against the proinflammatory recruitment of granulocytes to sites of apoptosis.

First discovered in milk almost 70 years ago, lactoferrin is a highly pleiotropic iron-binding glycoprotein closely related to transferrin that is a well-known constituent of exocrine secretions and secondary granules of neutrophils. It has many antiinflammatory and immunoregulatory properties, and the majority of these are not related to its ability to chelate iron, although some of its bacteriostatic features are dependent on iron binding (36, 37). Its ability to inhibit neutrophil migration, as described here, is an additional antiinflammatory feature of the molecule that is independent of its iron-chelating activity. In addition to its well-established antimicrobial properties, lactoferrin can inhibit proinflammatory responses not only through its ability to bind to key components of bacteria and viruses, but also through its direct activity on innate immune cells and molecules (36, 37). Thus, lactoferrin can inhibit production of proinflammatory mediators such as TNF-α and IL-6 (38, 39) and can promote production of antiinflammatory mediators including IL-10, IL-4, and TGF-β1 (40, 41).

The identification of specialized high- or low-affinity lactoferrin receptors on human neutrophils provides an initial mechanistic insight into how lactoferrin exerts its inhibitory effect on the neutrophil migration machinery. This is in accordance with our

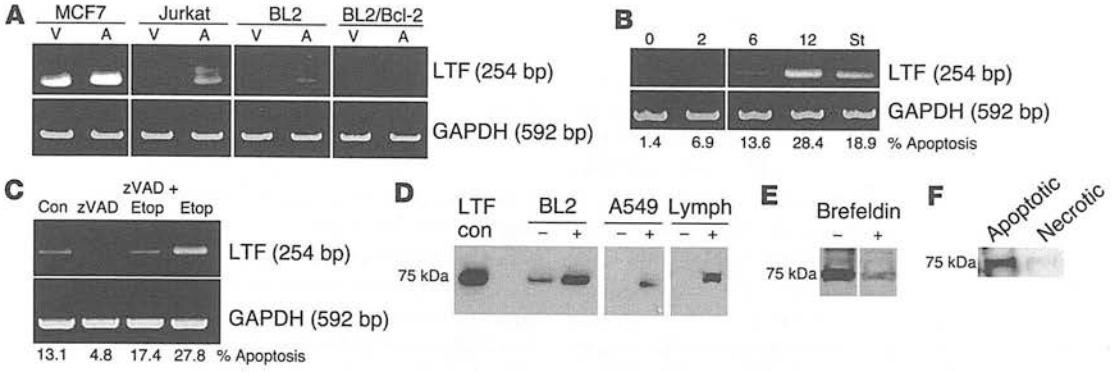

Figure 8

Induction of apoptosis upregulates lactoferrin expression and release. (**A**) RT-PCR analysis in cell lines stimulated to undergo apoptosis (A) and unstimulated controls (V). MCF7 cells transfected with caspase-3 (25.4% apoptosis; 100 μM etoposide, 20 hours), Jurkat (18.4% apoptosis; 1 μM staurosporine, 3 hours), BL2 (12.46% apoptosis), and BL2/Bcl-2 (7.42% apoptosis; 1 μM staurosporine, 1 hour). The lanes were run on the same gel but, where indicated by the vertical lines, were noncontiguous. (**B**) Lactoferrin expression in A549 cells at defined time points (hours) following stimulation with 100 μM etoposide or 1 μM staurosporine. (**C**) Addition of pan-caspase inhibitor zVAD-fmk (100 μg/ml) for 12 hours in order to prevent etoposide-induced apoptosis in A549 cells. (**D**) Immunoblot analysis of cell supernatants from: BL2 and primary lymphocytes in the presence (+) or absence (–) of staurosporine (1 μM) in serum-free conditions for 1 hour. A549 cells were stimulated with (+) or without (–) 100 μM etoposide for 5 hours. All samples were run on the same gel. Noncontiguous samples of A549 cells and lymphocytes (Lymph) are indicated by the vertical lines. (**E**) A549 cells were induced to become apoptotic (100 μM etoposide; 20 hours) in the presence or absence of brefeldin A (1 μg/ml), a protein release inhibitor. (**F**) Immunoblot analysis of cell supernatants from control BL2 cells (1×10^6/ml) induced to undergo apoptosis (1 μM staurosporine, 1 hour) or primary necrosis (56°C, 1 hour) in serum-free conditions. St, staurosporine; con; control; Etop, etoposide.

results demonstrating that lactoferrin binds directly to neutrophils and impinges on intracellular signaling events coupled to neutrophil activation, rather than interacting with chemoattractants and modulating their activity. Moreover, the latter possibility was further excluded by the fact that the observed inhibitory effect of lactoferrin was (a) specific to neutrophil, not monocyte, migration, (b) observed with a diversity of chemoattractants, and (c) not directly neutralized by chemoattractants. Consistent with other studies (26, 27), our results demonstrated 2 classes of lactoferrin receptor on the surface of human neutrophils. Our results indicate that a relatively high-affinity receptor is expressed at relatively low density and that a relatively low-affinity receptor is expressed at relatively high density. However, the detailed characterization of the lactoferrin receptor(s) and dissection of downstream signaling pathways remain a significant challenge, as lactoferrin has been extensively reported to interact with a range of molecules and receptors in a cell-specific manner and therefore has the potential to elicit different signaling cascades in different cell types. Indeed, many researchers over many years have attempted the detailed characterization of lactoferrin receptors from various cell types with only partial success, and many candidate receptors have been proposed for different cell types. Importantly, as has been suggested elsewhere (42), critical difficulties in the identification of specific lactoferrin receptors stem from the cationic nature of the lactoferrin protein, allowing it to bind to anionic molecules found in virtually all cell types.

Here, we have observed several effects of lactoferrin on cell activation with clear implications for migration, adhesion, and motility of neutrophils. The initial phase of neutrophil activation following agonist stimulation is characterized by a rapid increase in $[Ca^{2+}]_i$, which in turn triggers the activation of PKC as well as a range of calcium-dependent signaling pathways that control key neutrophil effector functions, including degranulation, cytoskeletal rearrangements, and cell migration (32, 43–45). Our data clearly suggest that the observed lactoferrin-mediated inhibitory effects on neutrophil chemotaxis could be attributed to modulation of molecules other than those involved in the $Ca^{2+}{}_i$ response following agonist stimulation. In addition, analysis of the expression of early activation markers such as CD62L and CD11b following stimulation with diverse neutrophil agonists, including PMA, a specific synthetic intracellular activator of PKC, further suggests that lactoferrin acts downstream or independently of the PKC pathway, affecting signaling cascade components of the later phases of neutrophil activation. This notion was strengthened by our observation that lactoferrin inhibited the phosphorylation of p44/42 (ERK1 and ERK2) MAPKs, indicating that its action is mediated, at least in part, via the MAPK pathway, a key pathway downstream of PKC with a crucial role in the regulation of cytoskeletal rearrangement and cell adhesion (46–48). The previously reported specific interaction of lactoferrin with the calcium-calmodulin complex (Ca^{2+}/CaM) provides further insight into the potential intracellular signaling targets of lactoferrin (49). The Ca^{2+}/CaM complex is formed following the increase in $[Ca^{2+}]_i$ and PKC activation and regulates the activity of a number of pathways involved in cell adhesion and migration, including the ERK1/2 kinases (50). Indeed, the use of specific inhibitors of calmodulin activity has been demonstrated to mimic the effect of lactoferrin – inhibition of neutrophil migration – reported here (51–53).

Lactoferrin has been shown previously to bind to receptors on mononuclear phagocytes and to inhibit proinflammatory responses via NF-κB (39, 54, 55). Taken together with the evidence that apoptotic cells can suppress proinflammatory responses of mononuclear phagocytes and can elicit antiinflammatory responses by these cells (10, 11), our results indicate that it is now reasonable to suspect that these effects are mediated, at least in part, by lactoferrin produced by apoptotic cells. However, the involvement of the NF-κB pathway in the inhibition of neutrophil migration seems unlikely, since lactoferrin-mediated inhibition of chemotaxis of

neutrophils treated with gliotoxin, a specific NF-κB inhibitor, was found to be identical to that of untreated cells (data not shown). In addition, no changes in the levels of IκB, an inhibitor of NF-κB that is degraded following NF-κB activation, were observed in Western blot analysis of fMLP-stimulated neutrophils treated with or without lactoferrin (data not shown).

In general terms, in professional phagocytes, sensing mechanisms that are not yet well defined allow these cells to navigate to apoptotic cells through chemotactic processes involving lipid signaling molecules and classical chemokine mechanisms (1, 2). It is noteworthy that of the 2 categories of professional phagocytes — mononuclear (monocytes and macrophages) and polymorphonuclear (granulocytes) — apoptotic cells attract mononuclear phagocytes selectively (12). Indeed, the lack of recruitment of granulocytes to apoptotic zones is a hallmark of the non-phlogistic apoptosis program, distinguishing normal areas of cell death from sites of accidental or pathological tissue damage or infection. Recent evidence suggests that certain recombinant preparations of lactoferrin have the potential to chemoattract monocytes (56). Although we failed to observe monocyte attraction to lactoferrin in our studies with milk-derived lactoferrin, these results indicate that lactoferrin has the potential to function in the selective attraction of monocytes but exclusion of granulocytes from sites of apoptosis. Our observation that lactoferrin, released into the supernatants of apoptotic cells, is able to inhibit the migration of neutrophils, demonstrates not only that physiological concentrations of lactoferrin are functional in mediating this effect but also that apoptotic cells are active in producing a factor that can potently suppress migration of neutrophils, the major subset of professional phagocytes among circulating leukocytes, to sites of apoptosis. Although it is clear that the secretory events inhibited by brefeldin A treatment (transport from the ER and Golgi network) are important for the release of lactoferrin from apoptotic cells, further work will be required to elucidate the details of this secretory process — including whether lactoferrin is transported directly from Golgi membranes for release from the cell surface.

While neutrophils may lack the sensing mechanisms to guide them to sites of apoptosis, the constitutive production of a factor that blocks neutrophil migration to such sites is likely to be important when the apoptosis program is activated in the presence of signals that promote neutrophil recruitment during inflammatory responses. In this context, it is noteworthy that lactoferrin is active in inhibiting IL-8 production by endothelial cells (57). Furthermore, lactoferrin's ability to inhibit neutrophil migration is likely to be important in the resolution of acute inflammation. Thus, our results suggest that lactoferrin arising from the secondary granules of neutrophils and from apoptotic cells constitutes a negative feedback component that limits the influx of neutrophils to inflammatory sites. We would speculate that neutrophils undergoing apoptosis en route to resolution of inflammation would release lactoferrin as do other apoptotic cells but would be unlikely to be required to synthesize the protein de novo. The negative regulatory activity of lactoferrin described here places it as one of the few molecules, alongside lipoxins, netrin-1, and annexin-1, that negatively regulate neutrophil migration (21, 58, 59). More importantly, based on the high specificity of its migration-inhibitory properties to neutrophils, lactoferrin is identified here as a promising therapeutic target for a range of chronic inflammatory conditions, including vasculitis, pulmonary fibrosis, and ischemia/reperfusion injury, that are characterized by excessive neutrophil infiltration leading to neutrophil-mediated host tissue damage and remodeling (19).

Since the properties of lactoferrin extend well beyond its ability to regulate infection, immune responses, and inflammation, the link established here between lactoferrin production and apoptosis has many additional implications. First, lactoferrin's ability to act as a growth factor (60, 61) extends evidence coupling the apoptosis program with tissue repair responses (62, 63). Second, additional recent evidence suggests that its protease activity may be instrumental in activating the caspase cascade. In particular, endogenous lactoferrin has been reported by 2 groups to induce apoptosis through activation of caspase-3 (64, 65). It is tempting to speculate that the de novo synthesis of lactoferrin by cells triggered to undergo apoptosis in our studies is linked to the initiation of the apoptosis program as well as to its antiinflammatory features. Therefore, lactoferrin may have multiple properties in apoptosis: (a) regulating the initiation of the program, (b) influencing repair in the tissue microenvironment, and (c) promoting the non-phlogistic nature of the process.

Lactoferrin's functions in relation to tumor biology are currently unclear. Proteolyzed forms of exogenous lactoferrin have been reported to have proapoptotic and antitumor activities. In addition, lactoferrin appears to promote antitumor host immune responses (37), and activation of antigen-presenting cells has recently been reported using high doses of recombinant lactoferrin produced in *Aspergillus* (56). Its observed effects on cell growth are inconsistent, and both growth-promoting and growth-inhibiting effects have been reported (60, 61, 66, 67), suggesting the possible importance of tissue context. Since lactoferrin has potent ability to inhibit neutrophil migration, it seems likely that in tumors in which neutrophils play a supportive role, limitation of neutrophil infiltration through lactoferrin administration could be therapeutically beneficial. However, in tumors in which neutrophils are absent, we propose that, given the known antitumor effects of neutrophils (68–71), encouragement of neutrophil infiltration through inhibition of lactoferrin may effect tumor destruction. Our results might predict that tumors in which apoptosis is prominent would produce lactoferrin in situ. While this suggestion requires proper investigation, at least in one category, BL, a high-grade malignancy in which high-rate apoptosis occurs, in situ production of lactoferrin protein has been known for many years (72).

In conclusion, we describe a novel homeostatic function for the highly pleiotropic immunomodulatory glycoprotein lactoferrin. We demonstrate that lactoferrin production is closely coupled to the fundamental cell death program, apoptosis. In this respect, lactoferrin is much more generally expressed than previously realized. Our results show that lactoferrin endows apoptotic cells with antiinflammatory properties, including the capacity to inhibit neutrophil migration. Given the multifunctional abilities of lactoferrin, these results have broad implications for the influence of apoptotic cells on multiple physiological processes, including cell growth, differentiation, and innate and adaptive immune responses, as well as the pathological processes of inflammatory and malignant diseases.

Methods

Antibodies and reagents. The following antibodies and reagents were used in this study: rabbit polyclonal anti-human lactoferrin IgG antibody (Sigma-Aldrich), mouse monoclonal anti-human lactoferrin antibodies (LF-2B8, AbD Serotec; imab75 and imab77, ImmunoSolv), rabbit polyclonal immu-

noglobulin (IgG) negative control (Dako), IgG1 isotype control (Sigma-Aldrich), allophycocyanin-conjugated (APC-conjugated) anti-CD11b (mIgG1; BD), FITC-conjugated anti-CD62L (mIgG1; AbD Serotec), purified human lactoferrin from milk (Sigma-Aldrich), purified human lactoferrin from neutrophils (Athens Research and Technology), recombinant human lactoferrin (Sigma-Aldrich), human recombinant apolactoferrin/hololactoferrin (ProSpec), purified human transferrin (Sigma-Aldrich), recombinant human TNF-α (R&D Bioscience), PMA (Sigma-Aldrich), fMLP (Sigma-Aldrich), C5a (Sigma-Aldrich), IL-8 (Sigma-Aldrich), LTB_4 (Sigma-Aldrich), brefeldin A (Sigma-Aldrich), staurosporine (Sigma-Aldrich), and gliotoxin (Sigma-Aldrich). For lactoferrin absorption chemoattractants, each chemoattractant was incubated with purified human milk lactoferrin (10 μg/ml), and a mouse monoclonal anti-human lactoferrin antibody (LF-2B8; 10 μg/ml) or isotype control was added. Antibodies were completely removed by BioMag goat anti-mouse IgG (QIAGEN) magnetic beads, and the efficiency of antibody removal was assessed by analysis of the preabsorbed chemoattractants by anti-mouse IgG ELISA (data not shown). Furthermore, the depleted antibody was readily recovered from the beads (data not shown).

Cell isolation. Fresh human venous blood was collected from volunteers according to the Royal Infirmary of Edinburgh (Scotland) Research Ethics Committee (approval 1702/95/3/11), and mononuclear and polymorphonuclear (PMN) leukocytes were isolated as previously described (73). Neutrophils represented more than 95% of isolated PMN cells. Monocytes (>90% $CD14^+$ cells) were positively selected from isolated mononuclear leukocytes using CD14 magnetic beads (Miltenyi Biotec). Human monocyte-derived macrophages were obtained following culture of monocytes for 6 days in Iscove's modified Dulbecco's medium (IMDM) containing 10% autologous serum.

Chemotaxis assay. In vitro leukocyte chemotaxis was measured following a well-established transfilter cell migration assay, as previously described (12) using polyvinyl uncoated Transwell inserts (5 μm pore size; Costar, Corning). Time of incubation (37°C; 5% CO_2) varied for cell type (neutrophils: 60 minutes; monocytes: 90 minutes; macrophages: 4 hours). Unless otherwise stated, lactoferrin was used at 10 μg/ml. Chemotactic agents included fMLP (100 nM; Sigma Aldrich), C5a (6.25 ng/ml; Sigma Aldrich), IL-8 (50 nM; R&D Systems), and LTB_4 (100 nM; Sigma Aldrich). For neutralization experiments, rabbit polyclonal anti-human lactoferrin antibody (Sigma Aldrich) or negative control (Dako) were used. Filters were observed using an inverted microscope (Zeiss Axiovert 25), and relative cell migration was determined by enumerating the number of migrated cells in 10 random high-power (×400) fields.

shRNA lentiviral transduction of BL cells. Lactoferrin expression was downregulated using shRNA lentiviral vectors (MISSION; Sigma-Aldrich). Briefly, lactoferrin-targeted shRNA lentiviral plasmids (pLKO.1-puro) were cotransfected with ViraPower Lentiviral packaging mix (pLP1, pLP2, pLP-VSV-G; Invitrogen) to 293FT cells using Lipofectamine LTX (Invitrogen). BL2 cells were transduced with the shRNA-expressing lentivirus, and stable cell lines were generated by selection with puromycin (2 μg/ml; Sigma-Aldrich).

RT-PCR analysis. Total RNA was extracted (RNeasy kit; QIAGEN) and reverse transcribed (2 μg) using SuperScript III RT (Invitrogen). Resulting cDNAs were used as template in PCR experiments at a concentration of 1 ng/50 μl of PCR mixture. The primers used were: forward lactoferrin (5′-TGTCTTCCTCGTCCTGCTGTTCCTCG-3′) and reverse lactoferrin (5′-CTGCCTCGTATATGAAACCACCATCAA-3′), forward GAPDH primer (5′-CGACAGTCAGCCGCATCTTCTTTTGCGTCG-3′) and reverse GAPDH primer (5′-GGACTGTGGTCATGAGTCCTTCCACGATAC-3′). Cycle parameters (lactoferrin: 40 cycles; GAPDH: 28 cycles) were: denaturation 94°C for 1 minute, primer annealing at 67°C (lactoferrin) or 50°C (GAPDH) for 1 minute, and extension at 72°C for 45 seconds, with a first denaturation step at 94°C for 7 minutes and a final extension at 72°C for 5 minutes. Purified PCR products (QIAquick gel extraction kit; QIAGEN) were sequenced to confirm validity by the Sequencing Service of the School of Life Sciences, University of Dundee, using Applied Biosystems BigDye 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequence analyzer.

Peritonitis model. All animal procedures were carried out under a UK Home Office Animals (Scientific Procedures) Act 1986 project licence. Mice (8- to 12-week-old female C57BL/6 mice; $n = 7$ per group) were injected i.p. with purified human lactoferrin or transferrin (500 ng in saline/0.1%; BSA Sigma Aldrich) or saline/0.1% BSA alone followed by a second i.p. injection with 1% thioglycollate (500 μl) or saline/0.1% BSA after 20 minutes. Recruited leukocytes were harvested after 4 hours by peritoneal lavage with ice-cold saline containing 2 mM EDTA. Harvested cells were counted using a NucleoCounter (ChemoMetec), which excluded nonnucleated cells. To determine the number of neutrophils ($Gr\text{-}1^+$), cells were immunolabeled with PE-conjugated anti-mouse Ly6-Gr-1 and counted using Flow-Count beads (Beckman Coulter).

Histology and immunohistochemistry. Six- to 10-week-old BALB/c SCID mice were injected i.p. with 10^7 BL2 cells. Tumors developed i.p. within 2 months of injection. Mice were sacrificed and tumors excised. For positive control, BALB/c mice were immunized with sheep red blood cells and spleens harvested and frozen 7 days after i.p. injection. Immunohistochemistry was performed on frozen acetone-fixed sections (5 μm) of BL or spleen tissues using biotinylated anti-mouse Gr-1 antibody (10 μg/ml; BioLegend) or isotype control (AbD Serotec). Nonspecific adsorption of antibodies was blocked using serum-free Protein Block (Dako). Reactions were amplified using VECTASTAIN Elite ABC avidin-biotinylated peroxidase complexes (Vector Laboratories). Hematoxylin was used as counterstain.

Flow cytometry. Unless otherwise stated, cells were suspended in PBS containing 5% normal mouse serum or 0.1% BSA, and all antibody incubations were performed for 20 minutes on ice. Mouse neutrophils were defined based on the expression of Gr-1 epitope using PE-conjugated rat anti-mouse Ly6G (Gr-1; eBioscience). For the assessment of neutrophil activation, the following antibodies were used: FITC-conjugated anti-CD62L (FMC46, mIgG2b; AbD Serotec) and APC-conjugated anti-CD11b (ICRF44, mIgG1; BD). Isotype controls included mouse IgG1:FITC (AbD Serotec), mouse IgG1:APC (BD), and rat IgG2b:PE (eBioscience). Cell apoptosis was determined by labeling with annexin V and propidium iodide. Samples were analyzed using a BD FACSCalibur or FACScan cytometer, and data were analyzed using BD CellQuest software.

Size fractionation and ion exchange chromatography. Size fractionation of BL2 cell conditioned media was performed using filters with specific molecular weight cutoff sizes (Amicon Centrifugal filters YM-50 and YM-100; Millipore), following the manufacturer's instructions. Ion exchange chromatography was carried out using Q Sepharose Fast Flow beads (Sigma-Aldrich). Beads were washed with neutralizing buffer, and bound proteins were then eluted by addition of 10 mM NaAc, 0.5 M NaCl, pH 4. BL2 cell–conditioned medium or control medium (RPMI 1640) was mixed with the beads and incubated at room temperature for 5 minutes. Samples were centrifuged (300 *g*, 5 minutes) and supernatants stored. Beads were washed with neutralizing buffer, and bound proteins were then eluted by addition of the corresponding elution buffer (for S beads: 10 mM Tris, 0.5 M NaCl, pH 10; for Q beads: 10 mM NaAc, 0.5 M NaCl, pH 4). After a 5-minute incubation at room temperature, beads were centrifuged (300 *g*, 5 minutes) and supernatants collected and analyzed. Prior to chemotaxis analysis, the supernatants were diluted (1:100), and pH was adjusted to 7.0. Proteins were identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry. The procedure was carried out by the Scottish Instrumentation and Resource Centre for Advanced Mass Spectrometry (SIRCAMS), School of Chemistry, University of Edinburgh.

Immunoblotting. Conditioned media from viable and apoptotic BL2 and A549 cells were collected, and their protein content was TCA precipitated. Briefly, 100 μl TCA was added in 1 ml conditioned medium at 4°C. Samples were centrifuged at 18,000 *g* and the pellets washed in ice-cold acetone before resuspension in sample buffer (NuPAGE; Invitrogen). Neutrophils (5×10^6 cells/ml) were lysed for 15 minutes with 1% NP-40 in TBS containing protease inhibitor cocktail (Sigma Aldrich), aprotinin, leupeptin, pepstatin A, 4-(2-aminoethyl)benzenesulfonyl fluoride, sodium orthovanadate, benzamidine, levamisole, and β-glycerophosphate. Samples were centrifuged (20,000 *g*, 4°C, 15 minutes) and resolved by SDS-PAGE using 4%–12% Bis-Tris gels (NuPAGE; Invitrogen). Proteins were then electroblotted onto a nitrocellulose membrane (NuPAGE; Invitrogen), blocked with 0.5% (for lactoferrin) or 3% BSA (for ERK), and probed with monoclonal mouse anti–human lactoferrin (1:100; LF.2B8; AbD Serotec) or mouse monoclonal anti-MAPK activated (diphosphorylated ERK1 and ERK2) antibody (1:1,000; Sigma-Aldrich) or polyclonal mouse ERK2 (1:1,000; Santa Cruz Biotechnology Inc.) or rabbit monoclonal anti-IκBα (74) (1:2,500; E130; Abcam) followed by HRP-conjugated goat anti-mouse IgG (1:2,000; Amersham) or HRP-conjugated goat anti-rabbit IgG (1:2,500; Dako) and visualized using ECL (GE Healthcare).

Binding studies. Biotinylation of human milk–derived lactoferrin was performed using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Thermo Scientific). Fresh neutrophils were exposed to biotinylated lactoferrin (10 μg/ml) at 37°C for 1 hour, after which the cells were washed and lysates prepared, resolved by SDS-PAGE, electroblotted, blocked with 0.1% PBS-Tween, and probed with HRP-conjugated streptavidin (Dako). For Scatchard analyses, ^{125}I-labeled human milk–derived lactoferrin, prepared using Pierce Iodination Reagent (Thermo Scientific), was added at a constant amount to fresh human neutrophils in the presence of increasing amounts of cold, unlabeled lactoferrin. After 30 minutes at 4°C, cells were washed 3 times before gamma counting.

Measurement of $[Ca^{2+}]_i$. Measurement of $[Ca^{2+}]_i$ was performed as previously described (75). Briefly, freshly isolated neutrophils were resuspended (10^7/ml) in HBSS (without Ca^{2+}/Mg^{2+}) and were incubated with 2 μM FURA 2/AM (Calbiochem) at 37°C for 30 minutes. The cells were washed twice, resuspended at 2×10^6/ml in HBSS (with Ca^{2+}/Mg^{2+}), incubated for an additional 30 minutes at 37°C in the presence of lactoferrin (10 μg/ml), and then stimulated with fMLP (1 nM or 10 nM). $[Ca^{2+}]_i$ levels were determined based on 340:380 nm dual wavelength excitation in a PerkinElmer luminescence spectrometer at 37°C with constant stirring. Calibration was performed after each experiment using Triton X (R_{max}) and EGTA (R_{min}) (R_{max} and R_{min} are the maximum and minimum 340:380 fluorescence ratios, respectively). [Ca2+]$_i$ was calculated based on the 340:380 nm fluorescence ratio.

Statistics. Results from multiple experiments are presented as mean ± SEM. One-way ANOVA was performed, followed by Bonferroni's post-hoc test. In all cases, *P* values of 0.05 or less were considered significant.

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Association of FcγRIIa (CD32a) with Lipid Rafts Regulates Ligand Binding Activity[1]

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Binding of Igs to myeloid cells via FcR is a key event in the control of innate and acquired immunity. FcγRIIa (CD32a) is a receptor for multivalent IgG expressed predominantly by myeloid cells, and its association with microdomains rich in cholesterol and sphingolipids, termed as lipid rafts, has been reported to be essential for efficient signaling. However, for many myeloid cell types, ligand binding to CD32a is suppressed by as yet undefined mechanisms. In this study, we have examined the role of CD32a-lipid raft interactions in the regulation of IgG binding to CD32a. Disruption of lipid raft structure following depletion or sequestration of membrane cholesterol greatly inhibited CD32a-mediated IgG binding. Furthermore, specific CD32a mutants, which show reduced association with lipid rafts (A224S and C241A), displayed decreased levels of IgG binding compared with wild-type CD32a. In contrast, constitutively lipid raft-associated CD32a (GPI-anchored CD32a) exhibited increased capacity for IgG binding compared with the full-length transmembrane CD32a. Our findings clearly suggest a major role for lipid rafts in the regulation of IgG binding and, more specifically, that suppression of CD32a-mediated IgG binding in myeloid cells is achieved by receptor exclusion from lipid raft membrane microdomains. *The Journal of Immunology,* **2009, 182: 8026–8036.**

Neutrophils represent the most abundant population of circulating leukocytes and mediate the earliest events of an inflammatory response to invading pathogens. In response to a number of distinct chemoattractant stimuli, these phagocytic leukocytes are rapidly recruited to the sites of infection, where they deploy a range of effector functions aimed at the destruction and eradication of pathogens (1). These functions include the activation of NADPH oxidase producing reactive oxygen intermediates (ROI),[3] the release of proteolytic enzymes and antimicrobial molecules, and the phagocytosis of invading pathogens (2). Recognition of pathogens is a key step in the initiation of all these processes and neutrophils have the capacity to recognize many diverse pathogens via the expression of specialized receptors for complement and pathogen-associated molecular patterns, including TLR and seven transmembrane spanning G protein-coupled receptors that recognize LPS, dsRNA, N-formylated peptides, and unmethylated CpG (3).

Neutrophils also express FcR that enable the recognition of diverse Ags via Ab-mediated molecular bridging, thereby linking the innate with the adaptive branches of immunity. FcR interaction with Igs has important biological consequences including —but not limited to—phagocytosis, Ab-dependent cellular cytotoxicity, degranulation, cytokine production, Ag presentation, and regulation of Ab production (4). A number of FcR have been identified and are categorized in terms of their interaction with Ab classes; particularly, FcγR, FcαR, FcεR, FcμR, and FcδR recognize and bind to IgG, IgA, IgE, IgM, and IgD, respectively.

IgG represents the most abundant Ab class present in circulation, and therefore, the role of FcγR has been extensively studied. Three classes of FcγR have been identified: CD64 (FcγRI), a high-affinity receptor for monomeric IgG and two receptors that, despite their low affinity for IgG monomers, bind particularly well to aggregated IgG via multivalent, high-avidity interactions, CD32 (FcγRII) and CD16 (FcγRIII) (5). Neutrophils constitutively express CD32a (FcγRIIa) and CD16b (FcγRIIIb) that are distinct in both their structure and function (6). In contrast, CD64 (FcγRI) expression is only induced following stimulation with IFN-γ (5). CD16b is a GPI-anchored membrane protein that lacks intracellular domains requiring accessory signaling proteins (e.g., γ- or ζ-chains) for the transduction of signals following receptor engagement. In contrast, CD32 represents the sole FcγR that contains distinct ITAM within the cytoplasmic domain. CD32a may therefore represent the exclusive neutrophil FcγR with the potential of transducing intrinsic signals upon engagement.

After recognition and binding of IgG complexes to CD32, the receptor becomes clustered and recruited to lipid raft microdomains (7–9). These regions of the plasma membrane, also termed detergent-resistant membranes (DRM) due to their characteristic insolubility following cold detergent extraction, act as docking sites for several intracellular signaling proteins. Association of a number of immunoreceptors with lipid rafts is necessary for receptor signaling and functional responses upon ligand binding

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[3] Abbreviations used in this paper: ROI, reactive oxygen intermediate; BxB, biotin anti-biotin immune complex; αCD, α-cyclodextrin; CHO, Chinese hamster ovary; DRM, detergent-resistant membrane; hHAIgG, human heat-aggregated IgG; HNE, human neutrophil elastase; MβCD, methyl-β-cyclodextrin; shRNA, short hairpin RNA; TfR, transferrin receptor; WT, wild type.

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(10), including AgR, such as BCR (11), TCR (12), adhesion molecules, like P-selectin glycoprotein ligand 1 (13), E-selectin (14), LFA-1 (15, 16), chemokine and cytokine receptors, such as CXCR4 (17), IL-2R (18), as well as key signaling proteins, including Ras (19), Kit (20), and Lyn (21) kinase.

It has been previously demonstrated that CD32a-lipid raft interactions are essential for efficient signaling events initiated following receptor cross-linking (22, 23). Mutations in key residues within the transmembrane region of CD32 that alter its association with lipid rafts have been reported to have a negative impact on its ability to transduce intracellular signals (24). Interactions of the ITAM of CD32 molecules with kinases of the Src and Syk family that preferentially reside within lipid raft microdomains lead to their phosphorylation and subsequently to downstream effector functions, including actin cytoskeletal rearrangements, initiation of respiratory burst, calcium mobilization, receptor endocytosis, and degradation (7, 9, 25).

Failure to control FcγR engagement would lead to the excessive release of neutrophil-derived cytotoxic compounds, including myeloperoxidase, hydrogen peroxide, elastase, and matrix metalloproteinases, that have destructive impacts on host tissue (26). Indeed, inappropriate neutrophil activation is associated with several chronic inflammatory conditions, such as vasculitis, rheumatoid arthritis, and glomerulonephritis, that are also linked to elevated levels of IgG complexes (27, 28). Thus, it is likely that regulatory homeostatic mechanisms exist to restrict IgG binding to neutrophils. For many myeloid cell types, including neutrophils, there is evidence that ligand binding to CD32 is suppressed, as evidenced by its poor interaction with IgG complexes (29, 30). However, the molecular mechanisms that account for this observed suppression still remain to be unraveled.

In this study, we hypothesized that the exclusion of CD32 from lipid raft microdomains might act as a regulatory mechanism that accounts for the observed suppression of CD32-mediated IgG binding to neutrophils. In this article, we have assessed the role of lipid rafts on IgG complex binding using chemical agents that alter lipid raft composition and structure. We have also examined the effect of specific mutations in the transmembrane and juxtamembrane regions of CD32 that displayed decreased association with lipid rafts on IgG complex binding. Furthermore, we generated a GPI-anchored CD32, which constitutively associated with lipid rafts and exhibited increased IgG complex binding when compared with the wild-type (WT) transmembrane receptor. Our findings clearly support a major role of lipid rafts in the regulation of IgG complex binding and, more specifically, that suppression of CD32-mediated IgG binding in myeloid cells is achieved by receptor exclusion from membrane domains.

Materials and Methods

Abs and reagents

All chemical reagents were obtained from Sigma-Aldrich and cell culture reagents were from Invitrogen and PAA laboratories, unless otherwise stated. Biotin-conjugated cholera toxin B subunit (CtxB) and Alexa Fluor 647-streptavidin were from Molecular Probes-Invitrogen. Human leukocyte elastase was obtained from Elastin Products Company. The following mouse anti-human CD32 mAbs were used in this study: IV.3 (IgG2b), AT-10 ($F(ab')_2$ from IgG1) and FLI8.26 (IgG2b, FITC-conjugated; BD Pharmingen). Goat polyclonal anti-human CD32a was obtained from R&D Systems. Purified mouse anti-human CD64 (10.1, IgG1) and CD16 (3G8, IgG1) were obtained from AbD Serotec. Corresponding isotype control Abs were from DakoCytomation, AbD Serotec, or Sigma-Aldrich. Mouse anti-human caveolin-I (C060, IgM and 2297, IgG1), mouse anti-human transferrin receptor/CD71 (clone 2, IgG1), mouse anti-human flotillin-1 (clone 18, IgG1), and mouse anti-phosphotyrosine (PY20, IgG2b) were from BD Transduction Laboratories (BD Biosciences). FITC- or R-PE-conjugated goat anti-mouse IgG $F(ab')_2$ fragments, HRP-conjugated goat anti-mouse Ig, and rabbit anti-goat Ig were from DakoCytomation. FITC-conjugated mouse anti-biotin (BN-34, IgG1) and purified human IgG were obtained from Sigma-Aldrich. Immune complexes were generated as described previously (29). Briefly, for biotin—anti-biotin complexes (BxB), FITC-conjugated anti-biotin (mouse IgG1; 170 $\mu g \cdot ml^{-1}$) was coincubated with biotin-conjugated BSA (500 $\mu g \cdot ml^{-1}$) at 4°C. Human IgG complexes (human heat-aggregated IgG; hHAIgG) were formed by incubating monomeric human IgG for 20 min at 63°C and centrifuged at 14,000× *g* to remove precipitates.

Cell isolation and culture

Ethical approval was obtained from the Lothian Local Research Ethics Committee, and polymorphonuclear leukocytes were isolated from peripheral venous blood of healthy donors as described previously (31). Briefly, after centrifugation of citrated whole blood (12.9 mM sodium citrate final concentration) at 350 × *g* for 20 min, platelet-rich plasma was removed, and erythrocytes were sedimented using 0.6% w/v Dextran T500 (Pharmacia). Polymorphonuclear leukocytes were separated from mononuclear leukocytes using discontinuous isotonic Percoll gradients (GE Healthcare) and were harvested from the 73%/61% interface. Purity was routinely assessed using morphologic criteria, and neutrophils represented >95% of the polymorphonuclear leukocyte population.

K562 and Jurkat cells (transfected with WT CD32a as described in Ref. 32) were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine (2 mM), and penicillin (100 $U \cdot ml^{-1}$)/streptomycin (100 $\mu g \cdot ml^{-1}$). Chinese hamster ovary (CHO-K1) cells were maintained in DMEM:F-12 (1:1) (Invitrogen) with GlutaMAX, 10% FCS, and penicillin/streptomycin. All cells were incubated at 37°C, 5% CO_2 in a humidified atmosphere.

Site-directed mutagenesis

Full-length human CD32a ORF subcloned into a pSELECT-neo-mcs vector was obtained from InvivoGen and was used as the template for the site-mutagenesis reactions that were performed based on the QuikChange Site II Kit (Stratagene). Mutated plasmid sequences were validated by direct sequencing, performed by the Sequencing Service (College of Life Sciences, University of Dundee) using Applied Biosystems Big-Dye 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

Short hairpin RNA (shRNA) mediated CD32 expression knockdown

CD32a expression was down-regulated using shRNA lentiviral vectors that were obtained from Sigma-Aldrich (MISSION shRNA clones). All five shRNA plasmid clones supplied were validated for CD32 knockdown efficiency by transient transfection to CHO cells expressing WT CD32a. One particular clone achieved >70% gene expression knockdown (clone TRCN029578; recognizing sequence 5′-GAAGAAACCAACAATGAC TAT-3′) and was stably transfected to WT K562 cells as described below. CD32a expression and IgG complex binding was assessed in shRNA expressing K562 cells by flow cytometry.

Generation of GPI-anchored CD32

The GPI modification site was predicted in the *CD55* gene based on Ref. 33 and the chimeric CD32/55 construct included the extracellular domains of CD32, followed by the GPI-anchored consensus sequence of CD55, comprising the ω site and downstream consensus domains (see Fig. 6*C*). CD32/55 protein was designed based on Ref. 34 (PreLink) to minimize misfolding of the chimeric protein. Sequence of the final construct was validated by direct sequencing, as described above. CD32/55 expressing plasmid was transfected in CHO-K1 and K562 cells, and the GPI modification of the chimeric protein was confirmed by reduction in expression following treatment with phosphatidylinositol-specific phospholipase C (Sigma-Aldrich) for 20 min at 37°C.

Cell transfection

CHO cells were transfected using either jetPEI Transfection reagent (Polyplus Transfection) or Lipofectamine LTX (Invitrogen), according to the manufacturer's instructions. K562 cells were transfected using Lipofectamine LTX and Lipofectamine Plus reagent (Invitrogen). For the generation of stable transfectants, CHO cells were cultured in DMEM:F-12 (10% FCS + GlutaMAX) medium containing 1 $mg \cdot ml^{-1}$ G418 (Invitrogen) for ~14 days. K562 cells were maintained in RPMI 1640 medium (10% FCS + 2 mM L-glutamine) and selected with either G418 (initially added at 1 $mg \cdot ml^{-1}$ for the first 48 h and then at 500 $\mu g \cdot ml^{-1}$) or

puromycin (2 $\mu g \cdot ml^{-1}$; Sigma-Aldrich). Selection of positive clones was performed by FACS using a BD FACSVantage SE/DiVa Cell sorter (BD Biosciences).

Flow cytometry

Immune complex binding was assessed based on a previously described assay (29) that measures binding of aggregated IgG to CD32, a low-affinity receptor via multiple binding sites that generate high-avidity interactions. Cells were incubated with the corresponding IgG complexes (BxB or hHAIgG) for 30 min on ice and under these conditions, IgG binding is essentially at equilibrium, with >70% of maximal IgG binding (20–60 min) observed after 5 min (data not shown). Similar IgG binding kinetics were also evident following MβCD treatment. For blocking experiments, function blocking anti-CD32 Abs (IV.3 or AT-10 $F(ab')_2$; 10 $\mu g \cdot ml^{-1}$) or anti-CD16 (3G8 $F(ab')_2$; 10 $\mu g \cdot ml^{-1}$) were used 10 min before the addition of IgG complexes. Analysis of FcR expression was performed by incubating the cells with the corresponding mouse mAbs (used at 10 $\mu g \cdot ml^{-1}$), followed by FITC-conjugated goat $F(ab')_2$ anti-mouse Ig (10 $\mu g \cdot ml^{-1}$; DakoCytomation). Flow cytometric analysis of the samples was performed using a BD FACSCalibur or FACScan cytometer (BD Biosciences). Data were analyzed using BD CellQuest (BD Biosciences) or FlowJo (Tree Star) software, and all results are presented as the median fluorescence intensity.

DRM fractionation and immunoblot analysis

DRM domain fractionation was performed essentially as previously described (8, 14) using well-validated protocols and all procedures were conducted at 4°C. Following stimulation of cells (CHO, 2×10^7, or K562, 1×10^7) with IgG complexes (hHAIgG), cells were washed with ice-cold PBS (Ca^{2+}/Mg^{2+} free) and incubated for 10 min with TNE buffer (50 mM Tris, 150 mM NaCl, and 2 mM EDTA (pH 7.5)) at 4°C. Then, cells were homogenized using a 25-gauge needle, and cell homogenates were incubated on ice for an additional 5 min, before the addition of Triton X-100 (0.25% v/v final concentration). Lysates were incubated for 30 min on ice, OptiPrep (Axis-Shield; Sigma-Aldrich) density gradient medium was added at a final concentration of 40%, and mixture (600 μl) was applied to the bottom of prechilled 2.2-ml ultracentrifuge tubes (Ultra-Clear; Beckman Coulter). On top, OptiPrep solutions (600 μl in TNE buffer) at concentrations of 35 and 25% were sequentially layered, followed by 200 μl of TNE buffer. Samples were centrifuged at 54,000 rpm (194,000 $\times$ g_{avg}) for 2 h at 4°C in an Optima-MAX benchtop ultracentrifuge using a TLS-55 rotor (Beckman Coulter). After centrifugation, fractions (200 μl) were collected and resolved by SDS-PAGE using 4–12% Bis-Tris gels (NuPAGE; Invitrogen), according to the manufacturer's instructions. Proteins were then electroblotted onto nitrocellulose membrane (Amersham Biosciences), blocked with 5% w/v fat-free milk, and probed with the corresponding primary Ab: goat anti-human CD32a (1/500), mouse anti-human caveolin-1 (1/500), mouse anti-human transferrin-receptor (1/1000), and mouse anti-human flotillin-1 (1/1000). Blots were then incubated with HRP-conjugated secondary Abs (either goat anti-mouse or rabbit anti-goat Ig; 1/5000) and visualized using ECL (Amersham Biosciences). For the quantification of gel-band intensities, ImageJ software package was used (National Institutes of Health).

Confocal immunofluorescence microscopy

CD32 and GM1 immunolabeling was performed using previously described protocols (35). Briefly, CHO cells were grown on sterile glass coverslips for 24 h (37°C; 5% CO_2) before immunolabeling. Cells were washed twice with ice-cold medium (DMEM containing 2 $mg \cdot ml^{-1}$ BSA) and incubated with the mouse anti-human CD32 Ab (IV.3 clone; 20 $\mu g \cdot ml^{-1}$) and biotin-conjugated CtxB (10 $\mu g \cdot ml^{-1}$) at 12°C for 60 min under gentle rocking. The specificity of CtxB for the GM1 ganglioside was validated by Western blot analysis of CHO and K562 cell lysates. Then, cells were washed with ice-cold PBS and subsequently incubated with Alexa Fluor 647-labeled streptavidin (10 $\mu g \cdot ml^{-1}$) and R-PE-conjugated goat $F(ab')_2$ anti-mouse Ig (10 $\mu g \cdot ml^{-1}$). Cells were fixed on ice for 4 min with 3.8% formaldehyde in PBS followed by a 5-min incubation in methanol at −20°C. K562 immunolabeling was performed as described above with the exception that cells were labeled in suspension and were then attached to poly-L-lysine (Sigma-Aldrich)-coated coverslips, according to the manufacturer's instructions before fixation. Coverslips were mounted using the Fluorsave reagent (Merck-Calbiochem), slides were visualized (×100 oil-immersion objective) using Zeiss LSM510meta laser scanning confocal microscope (Zeiss) equipped with argon and helium/neon lasers, and digital images were prepared using the LSM image browser (Zeiss) and Volocity (Improvision). For the quantification of CD32-GM1 colocalization, images (from at least 50 cells obtained from random fields) were analyzed using the Zeiss LSM 510 software package, or Volocity and colocalization is expressed as the percentage of pixels from the CD32 channel colocalizing with pixels from the GM1 channel.

Analysis of CD32 tyrosine phosphorylation

After stimulation of CHO cells with IgG complexes (10 $\mu g \cdot ml^{-1}$, 5 min, 37°C), cells (3×10^7/ml) were washed extensively in ice-cold PBS and lysed with 2% Triton X-100 in TBS, in the presence of phosphatase and protease inhibitors (Halt protease and phosphatase inhibitor mixture; Pierce/Thermo Scientific) for 15 min on ice, following by centrifugation (14,000 $\times$ g, 15 min, 4°C). Lysates were incubated with agarose-conjugated goat anti-mouse Ig (Sigma-Aldrich) for 60 min at 4°C to remove nonspecific protein interactions. Anti-CD32 Ab (IV.3) was then added to the precleared lysates and incubated for 60 min (4°C), following by goat anti-mouse Ig agarose for 90 min. Agarose pellets were extensively washed, and proteins were resolved by SDS-PAGE using 4–12% Bis-Tris gels (NuPAGE; Invitrogen), according to the manufacturer's instructions. Proteins were then electroblotted onto nitrocellulose membrane (Amersham Biosciences), blocked with 0.1% Tween 20, and probed with biotin-conjugated mouse anti-phosphotyrosine (1/7000). Blots were then incubated with HRP-conjugated streptavidin (1/5000) and visualized using ECL (Amersham Biosciences). To assess total CD32 content, membranes were stripped with 0.1 M glycine (pH 2.5) containing 0.1% Tween 20 and then blocked with 5% w/v fat-free milk and reprobed with goat anti-human CD32a (1/500), followed by HRP-conjugated rabbit anti-goat Ig (1/5000).

Statistical analysis

Unless otherwise stated, results from multiple experiments are presented as mean ± SD. One- or two-way ANOVA was performed followed by Bonferroni posthoc analysis. Values of $p \leq 0.05$ were considered to be statistically significant.

Results

Differential regulation of IgG binding to CD32 receptor in various cell types

To determine whether IgG complex binding to CD32 is suppressed in a cell context-dependent manner, we measured CD32-mediated IgG binding in diverse cell lineages, including K562, an erythromyeloid cell line, and CD32-transfected human Jurkat T cells (Jurkat:32) and Chinese hamster ovary (CHO:32) cells. Analysis of FcγR expression (CD64, CD32, and CD16) by flow cytometry revealed that CD32 was expressed by all the tested cell types (Fig. 1*A*). However, key differences were noted in terms of CD32-mediated IgG complex binding. In particular, although CD32 was expressed at substantial levels by neutrophils and K562 cells, IgG complex binding to these cells was essentially minimal. In contrast, CD32-transfected CHO and Jurkat cells displayed high levels of IgG complex binding (Fig. 1*B*; data not shown). Comparison of CD32-mediated IgG complex binding to K562 and Jurkat:32 cells, both cell types expressing CD32 at comparable levels, revealed that Jurkat cells were capable of IgG binding at much lower concentrations (Fig. 1, *C* and *D*). Similar analyses were performed in other cell types of the myeloid lineage, including monocytes, eosinophils, platelets, and HL-60, a myeloleukaemic cell line. In all these cell types, CD32 was expressed at substantial levels; however, IgG complex binding to CD32 was at negligible levels (data not shown). These results suggest the existence of common regulatory mechanisms that limit IgG binding to CD32 in cells of myeloid origin, such as neutrophils and K562 cells.

Involvement of lipid rafts in the regulation of IgG binding to CD32

Although the CD32-lipid raft association has been suggested to be required for the initiation of signal transduction events in response to receptor cross-linking, the role of lipid rafts in the regulation of IgG complex binding to CD32 is not clear. We therefore measured IgG complex binding to CHO:32 cells that were treated with a

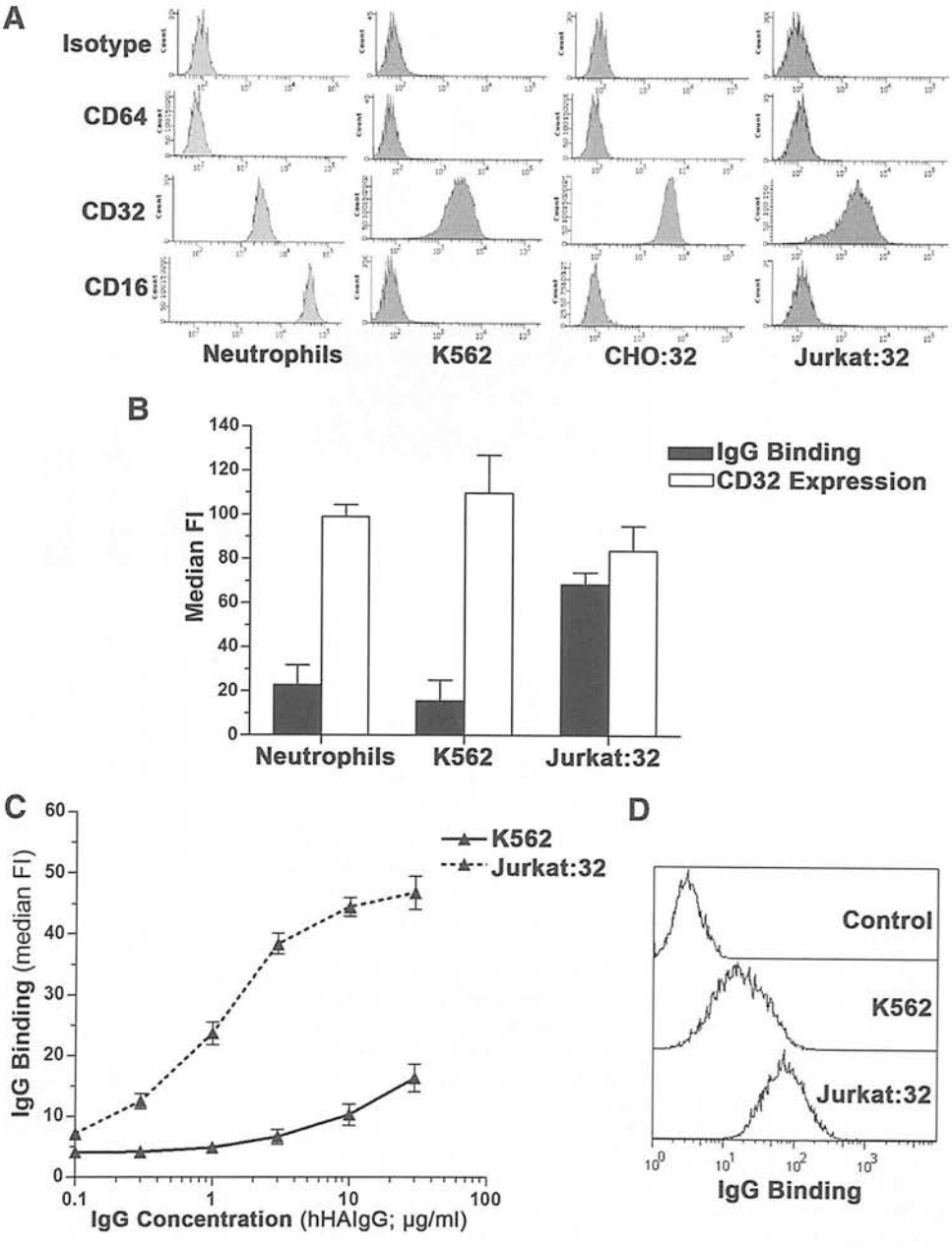

FIGURE 1. Differential regulation of IgG complex binding to CD32 receptor in various cell types. *A*, Representative flow cytometry histograms of FcγR expression in neutrophils, K562 cells, and in CD32-transfected CHO and Jurkat cells. *B*, Measurement of IgG complex binding (mIgG1; BxB; 10 $\mu g \cdot ml^{-1}$) and CD32 expression in neutrophils, K562 cells, and CD32-transfected Jurkat cells (Jurkat:32) were performed by flow cytometry as described in *Materials and Methods*. *C*, FITC-conjugated human IgG complex (hHAIgG) binding to K562 and Jurkat:32 cells over a range of IgG concentration was determined by flow cytometry. Results presented as the mean (±SD) from at least three independent experiments. *D*, Representative flow cytometry histograms of IgG complex binding (BxB; 10 $\mu g \cdot ml^{-1}$) to K562 or Jurkat:32 cells. Control indicates the profile of cells that were not coincubated with IgG complexes.

cholesterol-depleting agent, methyl-β-cyclodextrin (MβCD; 10 mM for 15 min at 37°C) to disrupt lipid raft microdomains. IgG complex binding to CD32 was greatly reduced following treatment with MβCD, whereas the inactive analog of MβCD, α-cyclodextrin (α-CD), had no impact on IgG binding (Fig. 2, *A–C*). In addition, cholesterol repletion (10 mM cholesterol-MβCD, 30 min, 37°C) in MβCD-treated cells abrogated any effects on IgG complex binding observed following MβCD treatment (data not shown). Further evidence in support of the requirement for intact lipid rafts for IgG complex binding to CD32 was obtained using nystatin, a chemical agent that binds to cholesterol, thereby altering lipid organization of the plasma membrane (Fig. 2*C*). In addition, reduced IgG complex binding to CD32 was observed following treatment of CHO:32 cells with short-chain ceramides (C2-ceramide and C6-ceramide) that also disrupt lipid raft organization, whereas the inactive form of C2-ceramide, C2-dihydroceramide, had no impact on IgG binding (Fig. 2*D*). It should be noted that disruption of lipid rafts had no effect on CD32 expression (Fig. 2*E*), and none of the treatments had any impact on cell viability (data not shown). In summary, these results clearly indicate that intact lipid raft microdomains are required for efficient IgG complex binding to CD32.

Although the precise characterization of membrane lipid rafts is a topic of great debate, it is widely accepted that at least part of these domains display insolubility following cold nonionic detergent extraction, due to their cholesterol-enriched, low-density lipid environment. For this reason, a well-validated, commonly used technique for the isolation of lipid rafts, also adopted in this study, is the fractionation of detergent-insoluble regions termed as DRM by ultracentrifugation with density gradient media. We analyzed the association of CD32 with lipid raft domains and as it is evident in Fig. 2, *F* and *G*, in the resting state, CD32 is predominantly distributed within the detergent-soluble membrane fractions characterized by the absence of caveolin, with a small percentage of CD32 to be detergent insoluble. However, following cross-linking of CD32 with IgG complexes (10 $\mu g \cdot ml^{-1}$; hHAIgG), we observed redistribution of CD32 to the caveolin-rich DRM fractions of the membrane. Taken together, these results suggest that CD32 translocation to lipid rafts is associated with efficient IgG binding.

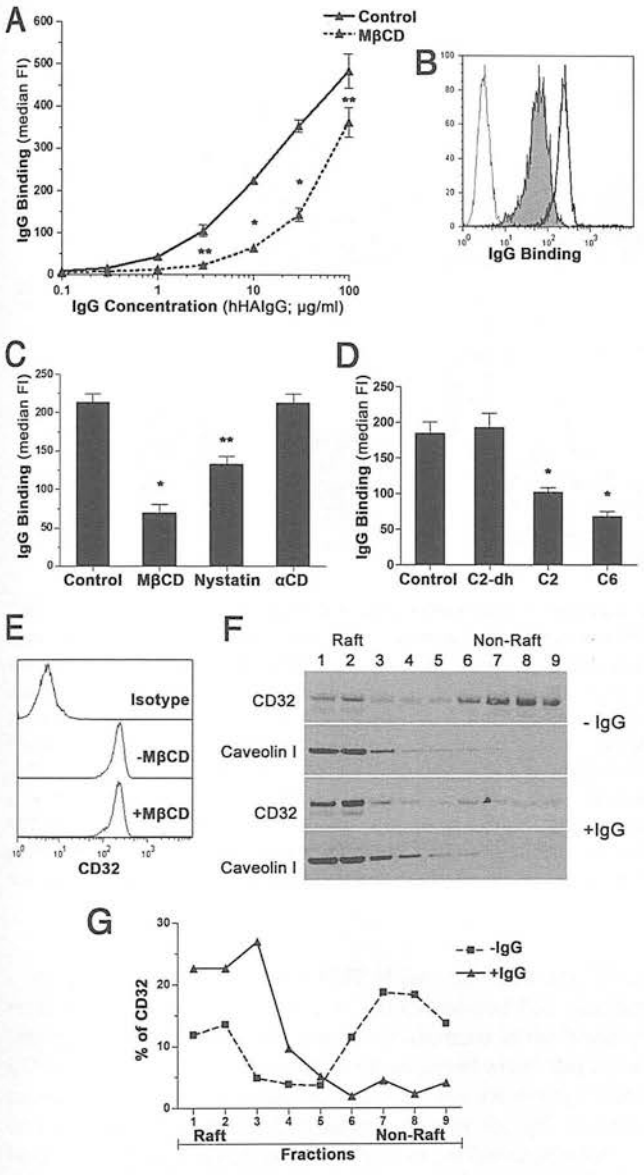

FIGURE 2. Involvement of lipid rafts in the regulation of IgG binding to CD32. *A*, Measurement of FITC-conjugated IgG complex binding (hHAIgG) to CD32-transfected CHO cells (CHO:32) following treatment with (MβCD) or without (control) MβCD (10 mM; 15 min, 37°C) (*, $p < 0.01$; **, $p < 0.05$). *B*, Representative flow cytometry histogram overlay of IgG binding to CHO:32 cells treated with (gray filled) or without (unfilled) 10 mM MβCD. *C*, IgG complex binding (hHAIgG; 10 $\mu g \cdot ml^{-1}$) to CHO:32 cells following disruption of lipid rafts by nystatin (30 $\mu g \cdot ml^{-1}$; 15 min, 37°C) or MβCD (10 mM; 15 min, 37°C). αCD (10 mM), an inactive analog of MβCD, was used as control. Results presented the mean (±SD) from at least three independent experiments (*, $p < 0.01$; **, $p < 0.05$) compared with untreated cells. *D*, Binding of human IgG complexes (hHAIgG; 10 $\mu g \cdot ml^{-1}$) to CHO:32 cells following treatment with short-chain ceramides: C2-ceramide (C2; 100 μM, 37°C, 60 min) and C6-ceramide (C6; 50 μM, 37°C, 60 min). Control indicates untreated cells, and C2-dihydroceramide (C2-dh; 100 μM, 37°C, 60 min) was used as the inactive form of C2 ceramide. Results presented the mean (±SD) from at least three independent experiments; *, $p < 0.01$ compared with untreated cells. *E*, Representative flow cytometry histogram of CD32 expression in control and in MβCD-treated CHO:32 cells. Isotype represents isotype-matched control. *F*, After incubation of CHO:32 cells with (+IgG) or without (−IgG) IgG complexes (hHAIgG; 10 $\mu g \cdot ml^{-1}$), DRM regions were extracted from cell lysates by fractionation using discontinuous OptiPrep gradient, as described in *Materials and Methods*. CD32 distribution was assessed by Western blot analysis of the fractions obtained. The constitutively lipid raft-associated protein caveolin-1 was used to define lipid raft fractions. *G*, Quantification of the percentage of CD32 present in each fraction was performed using ImageJ software.

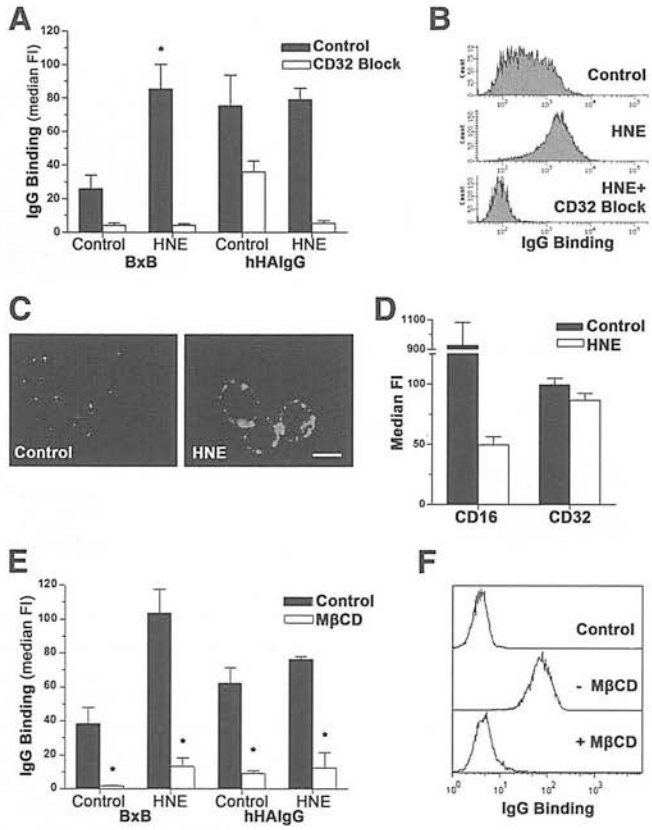

FIGURE 3. Elastase-mediated augmentation of IgG binding to neutrophils is dependent on lipid rafts. Binding of murine IgG1 (BxB; 10 $\mu g \cdot ml^{-1}$) or human IgG (hHAIgG; 30 $\mu g \cdot ml^{-1}$) complexes was measured in neutrophils following treatment with HNE (4 $\mu g \cdot ml^{-1}$; 20 min, 37°C). CD32-mediated IgG complex binding was determined using anti-CD32 function blocking Abs (AT-10 $F(ab')_2$; 10 $\mu g \cdot ml^{-1}$) (*A*, graphical representation; *B*, representative flow cytometry histograms). Results represent the mean (±SD) from at least three independent experiments; *, $p < 0.01$ compared with control cells. *C*, Representative confocal immunofluorescence microscopy micrographs of IgG binding to control and HNE-treated neutrophils. Scale bar: 10 μm. *D*, CD32 and CD16 expression was determined by flow cytometry in control (■) and HNE-treated (□) neutrophils. Results presented as mean (±SD) from at least three independent determinations. *E*, IgG complex binding was measured in control and HNE-treated neutrophils that were firstly incubated with (□) or without (■) 10 mM MβCD (15 min, 37°C). Results represent the mean (±SD) from at least three independent experiments; *, $p < 0.01$ compared with the corresponding control. *F*, Representative flow cytometry histograms of IgG complex binding (BxB) to HNE-treated neutrophils incubated with or without MβCD (10 mM; 15 min, 37°C).

Elastase-mediated augmentation of IgG binding to neutrophils is dependent on lipid rafts

We next assessed IgG complex binding to neutrophils treated with human neutrophil elastase (HNE), which has been previously shown to regulate several effector functions, including CD32-mediated IgG complex binding (36). We have determined the contribution of the two types of FcγR (CD32 and CD16; Fig. 1*A*) expressed by neutrophils in IgG complex binding, using function-blocking anti-CD32 and anti-CD16 Abs. In addition, since mIgG1 has specificity for CD32, we have used mIgG1 complexes (BxB) as a direct measure for CD32-mediated IgG binding. Blockade of CD32, but not of CD16, resulted in a complete inhibition of BxB binding demonstrating a specific binding to CD32 (Fig. 3*A*; data not shown), whereas both CD16 and CD32 contributed to human IgG complex (human heat-aggregated IgG; hHAIgG) binding (Fig. 3*A*).

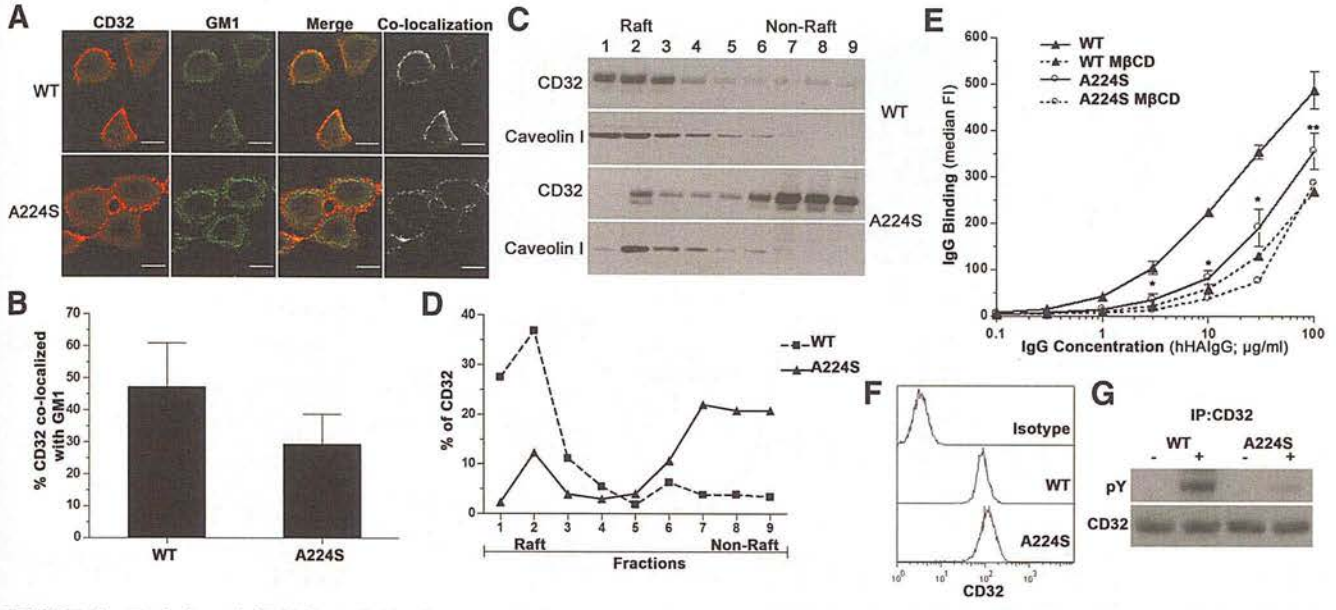

FIGURE 4. Exclusion of CD32 from lipid rafts results in decreased IgG binding in CD32-transfected CHO cells. Mutagenesis of A224S within the transmembrane region of CD32 resulted in decreased association of CD32 with lipid raft microdomains. *A* and *B*, Representative confocal immunofluorescence micrographs of CHO cells expressing CD32 (WT or A224S) and colocalization of CD32 with GM1, a marker for lipid raft domains together with quantification of CD32-GM1 colocalization (scale bar: 10 μm). Cells were prepared and analyzed as described in *Materials and Methods*. *C*, DRM fractionation and analysis of CD32 distribution in WT and A224S CD32-expressing CHO cells to determine receptor localization following IgG complex binding (hHAIgG; 10 $\mu g \cdot ml^{-1}$). Caveolin-1 was used as a marker for DRM fractions. *D*, Quantification of CD32 band intensities present in each fraction. *E*, Measurement of IgG complex binding (hHAIgG) in WT and A224S CD32-transfected CHO cells that were treated with or without 10 mM MβCD (15 min, 37°C). Results are presented as the mean from at least three experiments, and error bars indicate SD. **, $p < 0.05$, and *, $p < 0.01$, compared with WT CD32. *F*, Representative flow cytometry histogram of CD32 expression in WT and A224S CD32-expressing CHO cells. *G*, Analysis of tyrosine phosphorylation (pY) of WT and A224S CD32 following stimulation with IgG complexes (hHAIgG; 10 $\mu g \cdot ml^{-1}$, 5 min, 37°C). CD32 phosphorylation was analyzed as described in *Materials and Methods*, and as control, membranes were reprobed for CD32.

Treatment of neutrophils with HNE (4 $\mu g \cdot ml^{-1}$, 20 min, 37°C) resulted in a substantial increase in CD32-mediated IgG complex binding (Fig. 3, *A–C*) without significant alteration in the levels of CD32 (Fig. 3*D*). Similar effects were also observed when other serine proteases were used, including proteinase K (data not shown). These data suggest that proteases increase the capacity for IgG complex binding to CD32, independently of effects on surface expression.

Since disruption of lipid rafts resulted in a decreased IgG complex binding to cells that display constitutive IgG complex binding (CHO:32), we investigated whether the observed elastase-mediated augmentation of IgG binding to neutrophils was also dependent upon the presence of intact lipid raft microdomains. As shown in Fig. 3, *E* and *F*, IgG complex binding was also found to be markedly reduced following treatment of neutrophils with MβCD in elastase-treated neutrophils. Similarly, nystatin also displayed an analogous effect, with no change in IgG complex binding observed following cholesterol repletion in MβCD-treated cells or upon treatment with α-CD (data not shown). It should be noted that none of these treatments had any effect on neutrophil expression of CD32 (data not shown). Taken together, these data demonstrate that IgG binding to CD32 on neutrophils also required lipid raft integrity.

Exclusion of CD32 from lipid rafts results in decreased IgG binding

To further investigate the role of lipid rafts in the regulation of IgG complex binding, we examined a CD32 transmembrane mutation (A224S) that was previously shown to have impaired association with lipid rafts (24). We have generated the A224S CD32 mutation by site-directed mutagenesis and subsequently used transfection together with cell sorting to select a CHO cell population expressing levels of CD32 similar to control CD32-transfected cells. The association of the A224S mutant CD32 with lipid rafts was determined by confocal immunofluorescence microscopy and DRM fractionation. As it is evident in Fig. 4, *A* and *B*, the A224S mutant displayed decreased colocalization with the ganglioside GM1, a characteristic, well-established marker for lipid raft microdomains, compared with the WT receptor. Furthermore, unlike WT CD32, A224S failed to translocate to the detergent-insoluble fractions following cross-linking with IgG based on DRM fractionation analysis (Fig. 4, *C* and *D*). IgG complex binding were significantly reduced in A224S CD32-expressing CHO cells compared with WT CD32-CHO over a range of IgG concentrations, despite expressing CD32 at comparable levels (Fig. 4, *E* and *F*). More importantly, consistent with data shown in Fig. 2, treatment with MβCD had minimal impact on IgG complex binding to the A224S, indicating that the observed reduction in IgG binding could be attributed to the exclusion of this mutant receptor from lipid rafts. As receptor association with lipid rafts is a key determinant for the initiation of signaling cascades following receptor engagement, we have assessed and compared ITAM phosphorylation of WT and A224S CD32. As it is evident from Fig. 4*G*, reduced levels of phosphorylated CD32 were observed following IgG complex binding compared with the WT receptor, clearly indicating that exclusion of CD32 from lipid rafts could also have an impact on downstream signaling events.

Palmitoylation of CD32 promotes association with lipid rafts and regulates IgG binding

The CD32 protein has one potential site (C241) for palmitoylation, a posttranslational process that supports the association of membrane proteins with lipid rafts. This site was previously shown to be essential for CD32 palmitoylation and its association

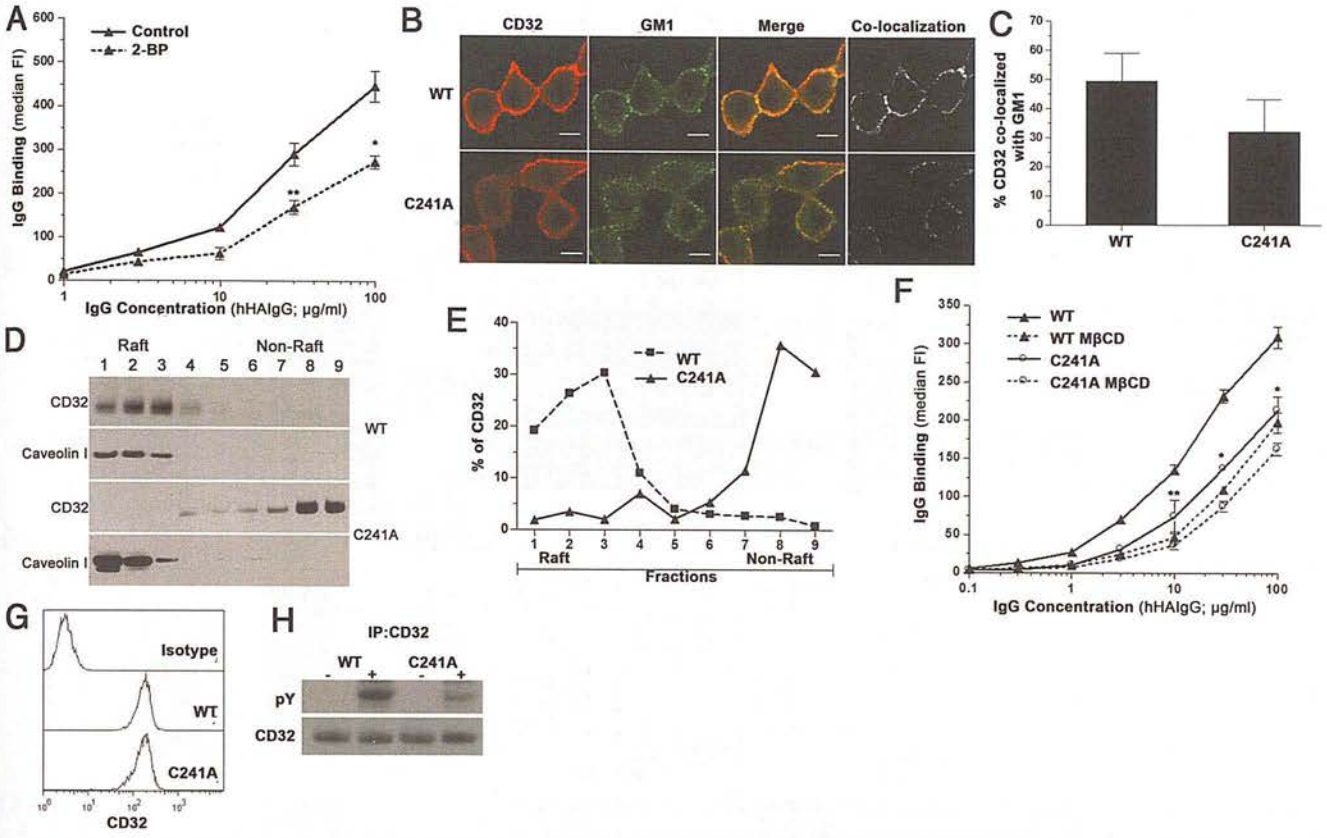

FIGURE 5. Palmitoylation of CD32 promotes association with lipid rafts and regulates IgG binding. *A*, FITC-labeled IgG complex binding (hHAIgG) was assessed in CD32-transfected CHO cells following treatment with 50 μM 2-bromopalmitate (20 h, 37°C), a palmitoylation inhibitor. Results represent the mean (±SD) from three independent experiments. *, $p < 0.01$, and **, $p < 0.05$, compared with untreated cells. Mutation of the CD32 palmitoylation site (C241A) resulted in decreased association of CD32 with lipid rafts, as evidenced by confocal immunofluorescence microscopy (*B* and *C*) and DRM fractionation analysis (*D* and *E*). *B*, Representative photomicrographs of CD32-GM1-labeled CHO cells expressing either the WT or the palmitoylation mutant (C241A) of CD32. Scale bar: 10 μm. *C*, Quantification of CD32 colocalization with GM1 in WT and C241A-expressing CHO cells. DRM fractionation (*D*) and quantification (*E*) of CD32 distribution in CHO cells transfected with either the WT or C241A CD32 upon binding of IgG complexes (hHAIgG; 10 $\mu g \cdot ml^{-1}$). Caveolin-1 was used to define DRM fractions. *F*, Comparison of FITC-conjugated IgG complex binding (hHAIgG) to WT and C241A CD32 following treatment with 10 mM MβCD (15 min, 37°C). Results are presented as the mean from three experiments and error bars represent SD. *, $p < 0.01$, and **, $p < 0.05$, compared with WT CD32. *G*, Flow cytometry histograms of CD32 expression in WT and C241A CD32-transfected CHO cells. *H*, Tyrosine phosphorylation (pY) of CD32 was assessed in CHO cells expressing either WT or C241A CD32 following stimulation with IgG complexes (hHAIgG; 10 $\mu g \cdot ml^{-1}$, 5 min, 37°C). CD32 phosphorylation was analyzed as described in *Materials and Methods* and to determine equal protein loading, membranes were reprobed for CD32.

with lipid raft microdomains (37). On the basis of our observation that exclusion of A224S CD32 from lipid rafts leads to a substantial decrease in IgG binding, we have next determined whether inhibition of CD32 palmitoylation had an impact on IgG binding.

First, the effect of the palmitoylation inhibitor 2-bromopalmitate on IgG binding to WT CD32-expressing CHO cells was assessed. Treatment with 2-bromopalmitate (50 μM, 20 h; 37°C) resulted in a significant decrease in IgG complex binding (Fig. 5*A*); an effect that was not associated with any changes in CD32 expression (data not shown), implicating palmitoylation of CD32 in the regulation of IgG binding. Second, mutation of the predicted palmitoylation site (C241A) of CD32 resulted in decreased CD32-GM1 colocalization in the CHO-C241A mutant compared with its WT counterpart (CHO:32) (Fig. 5, *B* and *C*). Unlike WT CD32, the C241A mutant also failed to distribute to the detergent-resistant domains in the presence of IgG complexes, suggesting possible exclusion from lipid rafts (Fig. 5, *D* and *E*). We next measured IgG binding in CHO-C241A cells, observing reduced levels of IgG complex binding when compared with CHO:32 WT cells (Fig. 5*F*), despite expressing CD32 at comparable levels (Fig. 5*G*). In addition, MβCD treatment of C241A had minimal effect on IgG binding, highlighting the role of CD32 palmitoylation in receptor–lipid raft association. In addition, when we assessed ITAM phosphorylation of CD32 following binding of IgG complexes, the C241A mutant displayed reduced levels of tyrosine phosphorylation compared with the WT receptor (Fig. 5*H*), further highlighting the role of lipid raft–CD32 association in the initiation of downstream signaling processes.

Constitutively lipid raft-associated CD32 displays increased IgG binding

Chemical disruption of lipid raft microdomains leads to decreased IgG binding to CD32 and mutations that alter the association of CD32 with detergent-insoluble domains have an analogous effect. Taken together, these findings indicate that translocation of CD32 to lipid rafts is a major process that regulates IgG binding in CHO cells, a cell type that displays unsuppressed IgG binding to CD32. We hypothesized that the low levels of IgG complex binding observed in cell types of myeloid origin, such as K562 cells, could be attributed to constitutive CD32 exclusion from lipid raft microdomains in these cell types. We therefore investigated whether constitutive localization of CD32 in lipid rafts would lead to an unsuppressed state in the myeloid environment of K562 cells.

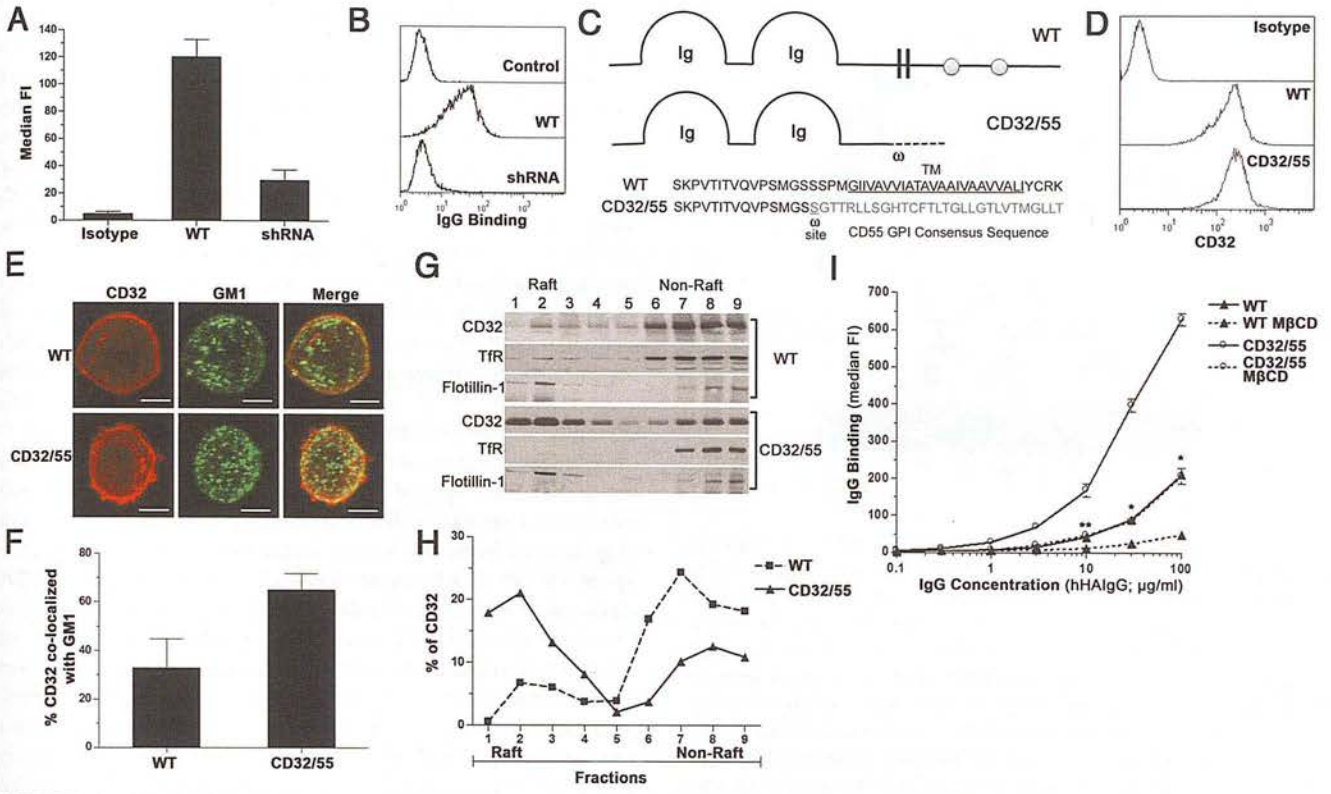

FIGURE 6. Constitutively lipid raft-associated CD32 displays increased IgG binding in K562 cells. *A*, Endogenous CD32 expression was knocked down in K562 following stable transfection with anti-CD32 shRNA-expressing plasmid vectors and surface expression was assessed by flow cytometry. Isotype represents isotype-matched control. Results are presented as the mean (±SD) from four independent experiments. *B*, Representative flow cytometry histograms of IgG complex binding (BxB; 10 $\mu g \cdot ml^{-1}$) to WT or shRNA-transfected K562 cells (shRNA). *C*, Schematic representation of a generated mutant form of CD32 with the extracellular domains of CD32, followed by the GPI modification consensus sequence of the GPI-anchored CD55 that is predicted to be primarily localized within lipid raft microdomains. *D*, Representative flow cytometry histogram of CD32 expression in shRNA-K562 cells that were stably transfected with either the WT, full-length CD32 (with synonymous mutations in the shRNA target region), or the CD32/55 chimeric construct. *E* and *F*, Analysis and quantification of CD32-GM1 colocalization in CD32 and CD32/55-expressing K562 cells by confocal immunofluorescence microscopy. Cells were prepared and analyzed as described in *Materials and Methods* and are presented as extended focus three-dimensional reconstructed images. Scale bar: 5 μm. *G*, DRM fractionation analysis of CD32 localization in WT or CD32/55 K562 cells revealed constitutive lipid raft association of CD32/55 but not of WT CD32. Transferrin receptor (TfR/CD71) and flotillin-1 distribution were assessed as markers for raft-excluded or raft-associated proteins, respectively. *H*, Quantification of the presence of CD32 in each fraction. *I*, Measurement of FITC-conjugated IgG complex binding (hHAIgG) in WT or CD32/55-transfected K562 cells treated with or without 10 mM MβCD (15 min, 37°C). Results represent the mean (±SD) from at least three independent experiments. *, $p < 0.01$, and **, $p < 0.05$, compared with CD32/55.

However, since K562 cells constitutively express CD32, we had to knock down endogenous CD32 expression in K562 cells using shRNA. K562 cells were stably transfected with plasmid vectors expressing shRNA targeted against CD32, resulting in >70% reduction in CD32 expression (Fig. 6*A*). Reduced expression of CD32 in these cells was associated with negligible levels of IgG complex binding (Fig. 6*B*). These cells (K562:CD32^{low}) were then stably transfected either with the WT CD32 (bearing synonymous mutations in the shRNA-recognizing sequence) or with a GPI-anchored version of CD32, which like all GPI-anchored proteins, was expected to be primarily localized to lipid raft membrane domains. This construct was engineered to comprise the extracellular domains of CD32, followed by the GPI modification consensus site of CD55 (Fig. 6*C*). Both WT CD32 and CD32/55 were expressed at similar levels (Fig. 6*D*) and the CD32/55 chimeric protein was found to be GPI-anchored, as evidenced by the reduction in expression following phosphatidylinositol-specific phospholipase C treatment (data not shown). Analysis of CD32/55 localization by confocal immunofluorescence microscopy revealed significantly higher colocalization with GM1 compared with the WT CD32 (Fig. 6, *E* and *F*). In contrast with WT CD32, which was excluded from rafts, CD32/55 was constitutively present in DRM fractions (Fig. 6, *G* and *H*). On the basis of these findings, we next investigated IgG binding to CD32/55 and to the WT counterpart. CD32/55 displayed markedly higher levels of IgG binding than WT CD32 (Fig. 6*I*). In addition, a great proportion of the IgG binding was inhibited following treatment with MβCD, suggesting that intact lipid rafts were necessary for the observed high levels of binding. In summary, all the presented data clearly indicate that in myeloid cells such as K562, CD32 is normally excluded from lipid raft microdomains, a process that accounts for the suppressed IgG binding observed.

Discussion

Several cell types of the myeloid origin, including monocytes and neutrophils are characterized by suppressed IgG complex binding to CD32, but the molecular mechanisms of suppression are poorly characterized. On the basis of our findings that disruption of lipid raft architecture using chemical agents, such as MβCD or nystatin, resulted also in decreased levels of IgG complex binding, we have tested the hypothesis that association of CD32 with lipid rafts are required for IgG complex binding and that exclusion of CD32 from lipid rafts acts to inhibit IgG complex binding to myeloid cells.

The heterogeneity of the plasma membrane comprising of variable-sized domains with distinct density and lipid composition can influence the activity and function of a range of membrane-bound proteins, controlling the interaction with downstream signaling components (10, 38). For this reason, the association of a protein with lipid rafts is a highly regulated, dynamic process, and several determinants for the control of this process have already been described, including the transmembrane domains. Interactions of the amino acid residues of these domains with complexes of cholesterol and sphingolipids present in lipid rafts influences the affinity for a protein for lipid rafts. Mutations within the transmembrane domains had an impact on their association with lipid rafts, possibly due to alterations in the overall three-dimensional structure (39).

For CD32, it has been demonstrated that alanine 224 within the transmembrane region of CD32 has a role on the receptor partitioning within the plasma membrane following cross-linking and mutation of this residue (A224S) resulted in decreased association with lipid rafts (24). Decreased levels of IgG complex binding to A224S-expressing CHO cells when compared with the WT receptor suggest that association of CD32 with lipid rafts is necessary for IgG complex binding. Association of CD32 with lipid rafts may be determined by a palmitoylation site that increases the affinity of CD32 for lipid rafts (37). We have clearly demonstrated that mutation of this site or inhibition of palmitoylation using chemical inhibitors results in decreased binding of IgG complexes; an effect that was attributed to the exclusion of CD32 from lipid rafts, further highlighting the role of lipid rafts in the regulation of IgG complex binding to CD32. The existence of a functional palmitoylation site clearly suggests that CD32-lipid raft interactions play a key role in the regulation of receptor activity and function. Although the available techniques for the study of lipid rafts have their respective strengths and weaknesses, in the present study, we have used two well-characterized approaches to study association of CD32 with lipid rafts: confocal immunofluorescence using CtxB that is known to specifically interact with the ganglioside GM1, a characteristic component of lipid rafts, as well as cold nonionic detergent extraction and fractionation. In addition, the efficiency of the DRM fractionation was validated by assessing the localization of known lipid raft associated (caveolin-1) and non-raft-associated (transferrin receptor) marker proteins.

Previous studies focusing on the importance of lipid raft domains in the regulation of CD32 effector functions have demonstrated that either chemical disruption of lipid rafts or mutants (in the transmembrane domain or in the palmitoylation site) with decreased affinity for lipid rafts impairs signaling responses (7–9, 22–24, 37). However, these studies did not measure binding of IgG complexes, and therefore, any impairment in receptor effector functions could be attributed to reduced ligand binding to CD32. Studies in which cross-linking of CD32 molecules with Abs or measuring the uptake of particles coated with anti-CD32 Abs as a surrogate of receptor engagement and activity may not represent a good model of the multivalent, low-affinity interaction of CD32 with IgG complexes, as they bypass normal physiological regulatory mechanisms.

A surprising observation of this study was the cell type-specific regulation of IgG complex binding to CD32. We have shown that in a range of myeloid cell types, CD32 has limited capacity for IgG complex binding, despite being expressed at levels, which confer significant binding in nonmyeloid cells. Since a constitutively lipid raft-associated form of CD32 (GPI-anchored CD32) results in high levels of IgG complex binding relative to WT CD32, we propose that CD32 is actively excluded from lipid rafts in myeloid cells. Given the role of lipid rafts in the regulation IgG complex binding

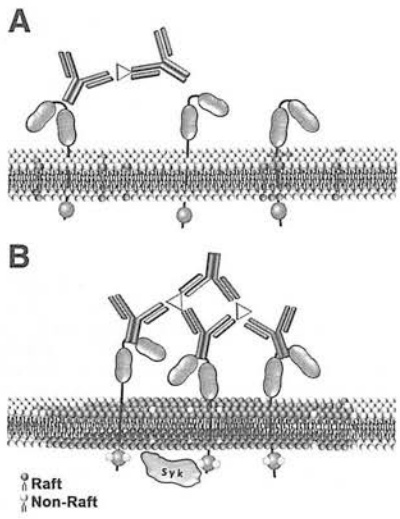

FIGURE 7. Schematic representation of the proposed model of control of IgG complex binding to CD32 by lipid raft domains. *A*, Under resting conditions, CD32 exists as a monomeric protein either being associated with small scale rafts or residing predominantly in the nonraft regions of the membrane. *B*, After recognition of IgG complexes, multiple low-affinity, high-avidity multivalent interactions occur between CD32 molecules and IgG complexes that induce receptor oligomerization, leading consequently to increased affinity of CD32 for lipid rafts and its redistribution to larger and more stable lipid rafts. During this process, the weak interactions between IgG and CD32 of the initial phase of the binding (*A*) are greatly stabilized following multivalent interactions and organization of CD32 to high-order lipid raft domains. Clustering of CD32 within these domains that are enriched with signaling molecules is essential for the interaction of the ITAM of CD32 with kinases (like Syk kinase) leading to the initiation of signaling cascades following receptor engagement.

we have already shown here, this exclusion may affect the stability of the CD32-IgG interactions. Cell type-specific association of molecules with lipid rafts has been previously described for the β_2 integrin, LFA-1 (15). LFA-1 functional activity is dependent on its association with lipid rafts and differential binding capacity for ICAM-1 was observed in monocytes and in dendritic cells (15, 16). The ability of LFA-1 to become organized in nanoclusters within lipid raft domains was suggested to augment LFA-1 affinity for ligand. We propose that a similar principle applies also for the regulation of ligand binding to CD32, which in myeloid cells is excluded from rafts and thus unable to oligomerize and associate with high-order lipid rafts, leading consequently to decreased stability of CD32-IgG complex interactions. On the basis of recent studies on the dynamics of lipid rafts, a model of lipid raft involvement in control of IgG complex binding could be proposed (Fig. 7) (35, 40). In the absence of IgG complexes, CD32 exists as a monomeric protein and is therefore associated with small-scale rafts or displays reduced affinity for rafts residing predominantly in the nonraft regions of the membrane with only a small fraction being raft associated (Fig. 2, *E* and *F*). After IgG complex binding to CD32, multiple low-affinity interactions with multivalent IgG complexes trigger receptor oligomerization and high-avidity binding, thereby stabilizing IgG complex binding to CD32. It is therefore highly possible that binding of IgG increases receptor localization to lipid rafts and as a consequence, lipid raft-associated CD32 would enhance IgG binding.

Several regulatory mechanisms could restrict the association of CD32 with lipid rafts in myeloid cells. Such mechanisms have been previously suggested to be responsible for the exclusion of proteins from lipid rafts and include the regulation of lipid membrane composition, protein palmitoylation, interaction of CD32

with membrane proteins with low affinity for lipid rafts or association of CD32 with cytoskeletal adaptor proteins, linking CD32 with nonraft domains of the membrane (40–43). Furthermore, it is likely that variations in the lipid composition of raft domains between different cell types might regulate the ligand binding activity of CD32. Preliminary analysis of the cholesterol content of lipid membranes of K562 and CHO cells revealed no major differences between these two cell types (K562, 3.7 ± 0.33 $\mu g/10^6$ cells; CHO, 4.2 ± 0.68 $\mu g/10^6$ cells). Similarly, previous studies on platelets and CHO cells—two cell types that display differential CD32 ligand binding activity—revealed similar cholesterol:phospholipid ratios (44, 45). Although it seems unlikely for the membrane cholesterol content to have a regulatory role in the IgG binding to CD32, the amount of specific phospholipid and glycolipid species within the lipid raft domains of different cell types might be an important determinant for the ligand binding activity of CD32, as well as for other raft-associated proteins.

Our data suggest that the mechanisms responsible for the exclusion of CD32 from lipid rafts are likely to be common in many myeloid cell types, including neutrophils, monocytes, and platelets, acting as an important regulatory component that limit IgG complex binding to these cells. Since myeloid cells are the first type of cells that encounter IgG complexes during an inflammatory response, it seems reasonable that lipid raft-mediated regulatory control of receptor activity has evolved to restrict IgG complex binding, preventing inappropriate cell activation by low levels of IgG complexes. The clustering of CD32 molecules within these domains that are enriched in signaling molecules is essential for the initiation of signaling cascades following receptor engagement.

There is increasing evidence of a role for lipid rafts in the regulation of other FcR activity. For instance, FcεRI binds monomeric IgE with high affinity and displays very low affinity for lipid rafts under resting conditions. Receptor activation following binding of oligomeric Ags cross-links two or more FcεRI receptors together, leading consequently to their recruitment into high-order lipid rafts, favoring phosphorylation of ITAM through interaction with raft-associated Lyn kinases or through the exclusion of phosphatases from lipid raft domains. CD64, the high-affinity receptor for IgG expressed predominantly in monocytes and macrophages, has also been reported to become constitutively associated with lipid rafts and changes in receptor partitioning to membrane microdomains may regulate IgG binding (46). Similarly, redistribution and colocalization of FcγRIIIa (CD16a) and IL-12R has been reported to be a key step in the initiation of intracellular signaling cascades, including ERK phosphorylation and subsequent effector functions, such as IFN-γ production following ligand binding (47). Furthermore, recent studies characterized a polymorphism (I232T) within the transmembrane domain of CD32b, an isoform of CD32 that contains an inhibitory ITIM with decreased affinity for lipid rafts and defective signaling (48, 49). Interestingly, increased frequency of the T232 polymorphism was noted in patients with systemic lupus erythematosus presumably because $CD32b^{T232}$ fails to localize to lipid rafts and therefore exert inhibitory activity upon autoimmune responses.

In conclusion, in this study, we have presented a novel regulatory role for lipid rafts, as an integral and important component for IgG complex binding to CD32. Indeed, disruption of lipid raft structure or alteration of receptor affinity for lipid rafts is associated with impaired IgG complex binding. In addition, we have shown that myeloid cells exhibit suppressed CD32 activity in terms of IgG complex binding, and this effect clearly arises from the active exclusion of CD32 from lipid rafts in these cells.

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Glucocorticoids Induce Protein S-Dependent Phagocytosis of Apoptotic Neutrophils by Human Macrophages[1]

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During resolution of an inflammatory response, recruited neutrophil granulocytes undergo apoptosis and are removed by tissue phagocytes before induction of secondary necrosis without provoking proinflammatory cytokine production and release. Promotion of physiological neutrophil clearance mechanisms may represent a viable therapeutic strategy for the treatment of inflammatory or autoimmune diseases in which removal of apoptotic cells is impaired. The mechanism underlying enhancement of macrophage capacity for phagocytosis of apoptotic cells by the powerful anti-inflammatory drugs of the glucocorticoid family has remained elusive. In this study, we report that human monocyte-derived macrophages cultured in the presence of dexamethasone exhibit augmented capacity for phagocytosis of membrane-intact, early apoptotic cells only in the presence of a serum factor. Our results eliminate a role for a number of potential opsonins, including complement, pentraxin-3, and fibronectin. Using ion-exchange and gel filtration chromatography, we identified a high molecular mass serum fraction containing C4-binding protein and protein S responsible for the augmentation of phagocytosis of apoptotic neutrophils. Because the apoptotic neutrophils used in this study specifically bind protein S, we suggest that glucocorticoid treatment of macrophages induces a switch to a protein S-dependent apoptotic cell recognition mechanism. Consistent with this suggestion, pretreatment of macrophages with Abs to Mer tyrosine kinase, a member of the Tyro3/Axl/Mer family of receptor tyrosine kinases, prevented glucocorticoid augmentation of phagocytosis. Induction of a protein S/Mer tyrosine kinase-dependent apoptotic cell clearance pathway may contribute to the potent anti-inflammatory effects of glucocorticoids, representing a potential target for promoting resolution of inflammatory responses. *The Journal of Immunology,* **2009, 183: 2167–2175.**

Successful restoration of a tissue to its original state after an inflammatory insult requires that large numbers of extravasated neutrophil granulocytes are cleared from the inflamed site. During this resolution phase of inflammation, recruited neutrophils undergo apoptosis and are subsequently removed by phagocytosis (1), a rapid and efficient process that does not stimulate proinflammatory macrophage responses (2). Conversely, inefficient or defective clearance of membrane-intact apoptotic neutrophils may result in release of their histotoxic intracellular contents as a consequence of secondary necrosis, potentially causing local tissue damage and contributing to pathogenesis of inflammatory disease (3). An attractive approach for therapeutic intervention in inflammatory diseases would therefore be to manipulate the processes involved in physiological clearance of neutrophils from inflamed sites. Although promotion of neutrophil apoptosis may be achievable pharmacologically (4), under some circumstances in vivo it will be important to ensure that the capacity for apoptotic cell clearance within tissues is matched to avoid potential deleterious consequences of the presence of non-phagocytosed apoptotic cells (5).

We have previously reported that the powerful anti-inflammatory drugs of the glucocorticoid family (methylprednisolone, hydrocortisone, or dexamethasone (Dex))[3] specifically enhance noninflammatory phagocytosis of apoptotic cells by human and murine macrophages (6, 7). Glucocorticoids have been shown to modulate the expression of over 100 genes, including those known to be associated with apoptotic cell phagocytosis, such as CD163, FPR1, and Mer tyrosine kinase (Mertk) receptors and MFG-E8 and C1q serum proteins (8). Furthermore, we have shown that human monocytes differentiated for 5 days in the presence of glucocorticoids exhibit a more homogeneous phenotype with reduced phosphorylation of molecules involved in integrin signaling and cytoskeletal rearrangement (7). However, the precise mechanism(s) by which glucocorticoids augment phagocytosis of apoptotic cells has remained elusive.

In this study, we have examined the mechanism underlying augmentation of human monocyte-derived macrophage (MDMϕ) capacity for phagocytosis of early membrane-intact apoptotic human neutrophils following exposure to glucocorticoids. Apoptotic neutrophils display a distinct surface molecular phenotype important for attenuation of functional responses (9) with additional surface changes that target dying cells for removal by phagocytes (10). A number of soluble factors present in serum, including complement C1q and C3b, properdin, collectins, long pentraxin-3, MFG-E8, galectin-3, and α_2-macroglobulin, have been reported to bind to apoptotic human cells (11–18) and consequently modulate their

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[3] Abbreviations used in this paper: Dex, dexamethasone; C4BP, C4-binding protein; CMFDA, 5-chloromethylfluorescein diacetate; MDMϕ, monocyte-derived macrophage; Mertk, Mer tyrosine kinase.

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recognition and uptake by macrophages via a number of different surface receptors, including scavenger receptors, complement receptors, receptors for phosphatidylserine, and Mertk (19–22). However, it is important to note that some of these opsonization events occur relatively late during the apoptotic process and accompany loss of membrane integrity (23).

In this study, we demonstrate that glucocorticoid augmentation of MDMϕ phagocytosis is associated with a switch from a serum-independent to a serum-dependent apoptotic cell recognition mechanism, which can be recapitulated with purified protein S, a 75-kDa vitamin K-dependent anticoagulation factor that is present in plasma at a relatively high concentration of ~25 μg/ml (24), and involves macrophage Mertk, a member of the Tyro3/Axl/Mer family of immunoregulatory receptor tyrosine kinases (25). Our data strongly suggest that glucocorticoids critically regulate a switch in apoptotic cell clearance mechanisms used by macrophages, potentially contributing to their potent anti-inflammatory effects and thus representing a target for promoting inflammatory resolution.

Materials and Methods

Sera, serum proteins, and other reagents

All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. Culture medium (IMDM), buffers (HBSS and PBS without divalent cations), and trypsin-EDTA were from PAA Laboratories. Percoll was from GE Healthcare. Dextran T500 was from Pharmacosmos. Dex was obtained from Organon. Roscovitine was from Merck. Serum, from coagulated whole blood, was obtained by cardiac puncture from wild-type, annexin I-deficient (26), and C1q-deficient (27) mice on a C57BL/6 background. C1q-depleted human serum was obtained from Merck. A soluble recombinant form of human complement receptor 1 was used for inhibition of C3 activation in serum (28). A dose of 250 μg/ml completely blocks complement activity, as assessed by hemolytic assays. Proteins purified from human serum/plasma were obtained from the following sources: protein S (Enzyme Research Laboratories), C1q (Merck), and α_2-macroglobulin (Sigma-Aldrich).

Antibodies

Primary Abs were from the following sources: polyclonal rabbit anti-protein S Ab (1:5000; DakoCytomation), anti-human Mer mAb (clone 125508, murine IgG2b, 1:50; R&D Systems), and CD44 mAb (clone 5A4, IgG1, 1:50; provided by G. Dougherty, University of California, San Francisco, CA). Control mouse Igs (IgG1, IgG2b; 1:50) were from Serotec. HRP-conjugated goat anti-rabbit Igs (1:2500) and FITC-conjugated F(ab')$_2$ goat anti-mouse Igs (1:50) were from DakoCytomation. PE-conjugated anti-CD16 mAb (clone 3G8, IgG1) was obtained from BD Biosciences. Agarose-coupled goat anti-rabbit Ig was obtained from Sigma-Aldrich.

Cell isolation

Mononuclear and polymorphonuclear leukocytes were isolated from freshly drawn, citrated human blood by dextran sedimentation and centrifugation over a discontinuous Percoll gradient (final concentrations of 55, 70, and 81% Percoll), as previously described (29). Mononuclear cells were aspirated from the 55/70% interface, and neutrophils from the 70/81% interface. Autologous serum was prepared by recalcification of platelet-rich plasma (final $CaCl_2$ concentration: 22 mM), as previously described (24).

In vitro culture of human MDMϕ

Mononuclear cells were resuspended at 4×10^6/ml in IMDM and adhered to 48-well tissue culture plates for 1 h at 37°C in 5% CO_2. Nonadherent lymphocytes were removed by washing with IMDM, and adherent monocytes were cultured for 5 days in IMDM containing 10% autologous serum ± 1 μM Dex. These cells are >90% CD14$^+$ at 5 days with functional and phenotypic characteristics of macrophages (Dex-MDMϕ) (7).

In vitro culture of neutrophils to induce apoptosis

Neutrophils were cultured at 4×10^6/ml in IMDM either in the absence of serum or in the presence of 10% autologous serum at 37°C in 5% CO_2 atmosphere for 20–24 h, during which time a proportion of the cells underwent apoptosis (1). Apoptosis and secondary necrosis were determined by annexin V-FITC binding (Roche Applied Sciences) and propidium iodide staining (Sigma-Aldrich), respectively. Alternatively, neutrophils were resuspended in IMDM at 2×10^7/ml and labeled with the fluorescent cell tracker dye 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen), 2 μg/ml for 15 min at 37°C in 5% CO_2. Neutrophils were then washed and cultured for 20–24 h, as described above.

Apoptotic cell phagocytosis assay

Phagocytosis of apoptotic cells was assessed essentially as described (30), using a method that has been carefully characterized and shown to discriminate between bound and internalized apoptotic cells, comparing favorably with microscopy analysis. CMFDA-labeled apoptotic neutrophils were centrifuged at 200 × *g* and resuspended at 2.5×10^6/ml in IMDM, and 0.5 ml was overlaid onto MDMϕ that had been cultured in 48-well plates (~200,000 MDMϕ/well, a ratio of ~10 neutrophils per macrophage) and then coincubated for 30 min at 37°C in 5% CO_2. Medium was then gently aspirated from the wells, and all cells were detached with 500 μl of trypsin-EDTA before determination of phagocytosis (percentage of FL-1-positive MDMϕ, identified by their distinct forward and side scatter properties) by flow cytometry using a FACScan (BD Biosciences). For assays conducted in the presence of serum or purified proteins, neutrophils were resuspended in IMDM containing either 1% autologous serum, 10 μg/ml either purified protein, or 1 mg/ml protein fractions from ion-exchange or gel filtration chromatography, unless otherwise stated in the figure legends. For experiments requiring preincubation with either Abs or other protein fractions from ion-exchange or gel filtration chromatography, MDMϕ or neutrophils were incubated with saturating concentrations of Abs (final concentration of ~10 μg/ml as determined by flow cytometric analysis) or 10 μg/ml either purified protein or 1 mg/ml protein fractions from ion-exchange or gel filtration chromatography, unless otherwise stated in the figure legends. Cells were washed and resuspended in IMDM before use in phagocytosis assays.

Serum fractionation

Human serum was dialyzed against 50 mM HEPES buffer (pH 7.0) containing 0.14 M NaCl overnight before anion-exchange chromatography using Q Sepharose (Sigma-Aldrich). Proteins were eluted using 50 mM HEPES containing 0.2 M NaCl, and fractions containing the highest amount of protein were combined before dialysis against 50 mM Tris (pH 7.4) containing 0.14 M NaCl (TBS) and concentrated before gel filtration chromatography using Sephacryl S-300 (GE Healthcare). The protein concentration in eluted fractions was estimated by measurement of absorbance at 280 nm (A280) using a spectrophotometer or using a bicinchoninic acid protein assay kit, as specified by the manufacturer (Pierce). Gel filtration fractions containing phagocytic activity were analyzed by SDS-PAGE, immunoblotting, and mass spectrometry to determine the proteins present (two separate analyses; J. Creanor, University of Edinburgh, Edinburgh, U.K.).

Immunodepletion and Western blotting

The 0.2 M NaCl eluate from anion-exchange chromatography was incubated with protein S Ab (5 μg/ml eluate) for 1 h on ice. Immunodepletion was achieved by incubation for 1 h with agarose-coupled goat anti-rabbit IgG at 4°C on a rotary mixer, followed by centrifugation at 13,000 × *g* for 1 min to pellet the agarose. To ensure efficient protein depletion, the supernatant was subjected to three rounds of depletion. Samples were resolved by SDS-PAGE using 9% gels under nonreducing conditions, unless otherwise stated, and transferred electrophoretically (80 V for 50 min) onto either polyvinylidene difluoride or nitrocellulose membranes (Millipore). Membranes were blocked overnight in TBS containing 0.1% Tween 20 before probing with Abs. Binding of anti-protein S Ab was detected with HRP-conjugated goat anti-rabbit Igs together with ECL (GE Healthcare).

Flow cytometry

All incubations were performed on ice to prevent internalization of Ab. Adherent MDMϕ were detached by incubation in HBSS without divalent cations containing 0.1% BSA and 3 mM EDTA. After washing with ice-cold HBSS containing 2% FBS, cells (10^5/assay) were incubated with saturating concentrations of mAb for 30 min. Cells were then washed twice in HBSS containing 2% FBS before incubation with FITC-conjugated F(ab')$_2$ goat anti-mouse Igs for 30 min before analysis using a FACScan flow cytometer (29).

Analysis of results

Results are presented as mean ± SEM, and n = number of independent experiments using macrophages from different donors. Results were analyzed by repeated measures one-way ANOVA with a Bonferroni posttest.

Results

Glucocorticoid-augmented clearance of apoptotic neutrophils by macrophages is serum dependent

Previous studies relating to glucocorticoid-enhanced phagocytosis of apoptotic cells used monocyte-derived macrophages and apoptotic cell targets that had been cultured in the presence of serum (6, 7). To evaluate the potential role of serum opsonization in promoting apoptotic neutrophil clearance, human blood monocytes were cultured for 5 days in the absence or presence of Dex, and neutrophils were rendered apoptotic by overnight culture in serum-free conditions (Fig. 1*A*). Neutrophil populations cultured in serum-free conditions for 20 h exhibit a slightly higher percentage (63–70%, n = 35, 95% confidence limit) of annexin V^+/propidium $iodide^-$ (apoptotic) cells when compared with neutrophils cultured in the presence of serum (50–60%, n = 10), consistent with the presence of a survival factor(s) in human serum. In addition, there were significantly higher percentages of annexin V^+/propidium $iodide^+$ (secondarily necrotic) neutrophils in serum-free cultures (18–26%, n = 35) when compared with neutrophils cultured in the presence of serum (8–17%, n = 10).

When we determined the proportion of untreated MDMϕ and Dex-MDMϕ that were capable of phagocytosis of neutrophils, we were surprised to find that there was no significant augmentation of phagocytosis of serum-free apoptotic neutrophils observed for Dex-MDMϕ (Fig. 1, *C* and *E*). In contrast, in the presence of 10% autologous serum, we observed increased phagocytic capacity for Dex-MDMϕ (Fig. 1, *D* and *E*). The presence or absence of serum also had a small, but statistically significant stimulatory effect upon phagocytosis of apoptotic neutrophils by untreated MDMϕ (Fig. 1*E*). The effect of serum on phagocytosis by Dex-MDMϕ was concentration dependent and reached significance at 1% (data not shown). The possibility that the presence of serum acts to promote phagocytic activity of MDMϕ directly was excluded in a series of experiments in which preincubation of apoptotic neutrophils with serum was found to confer augmentation of Dex-MDMϕ phagocytic capacity (data not shown), raising the possibility that a serum factor binds to the apoptotic neutrophil surface to promote phagocytosis by Dex-MDMϕ.

Augmentation of phagocytosis by serum is independent of the presence of necrotic neutrophils

Neutrophils undergo apoptosis in a relatively heterogeneous manner during in vitro culture (Fig. 1*A*), making it difficult to determine whether enhanced phagocytosis following opsonization depends upon the presence of apoptotic or secondarily necrotic cells. We therefore treated neutrophils with roscovitine, a cyclin-dependent kinase inhibitor that induces neutrophil apoptosis rapidly and uniformly without induction of secondary necrosis (4). Neutrophil populations cultured with 20 μM roscovitine in serum-free conditions for 4 h exhibit a high percentage (80–90%) of annexin V^+/propidium $iodide^-$ (apoptotic) cells with less than 3% annexin V^+/propidium $iodide^+$ (secondarily necrotic) cells (Fig. 1*B*). Serum-dependent enhancement of phagocytosis of roscovitine-treated apoptotic neutrophils by Dex-MDMϕ confirmed that opsonization of early apoptotic cells was required (33.7 ± 9.3% and 51.8 ± 6.3% for Dex-MDMϕ in the absence and presence of serum, respectively). Data are mean percentage phagocytosis ± SEM, n = 3 (**, $p < 0.01$).

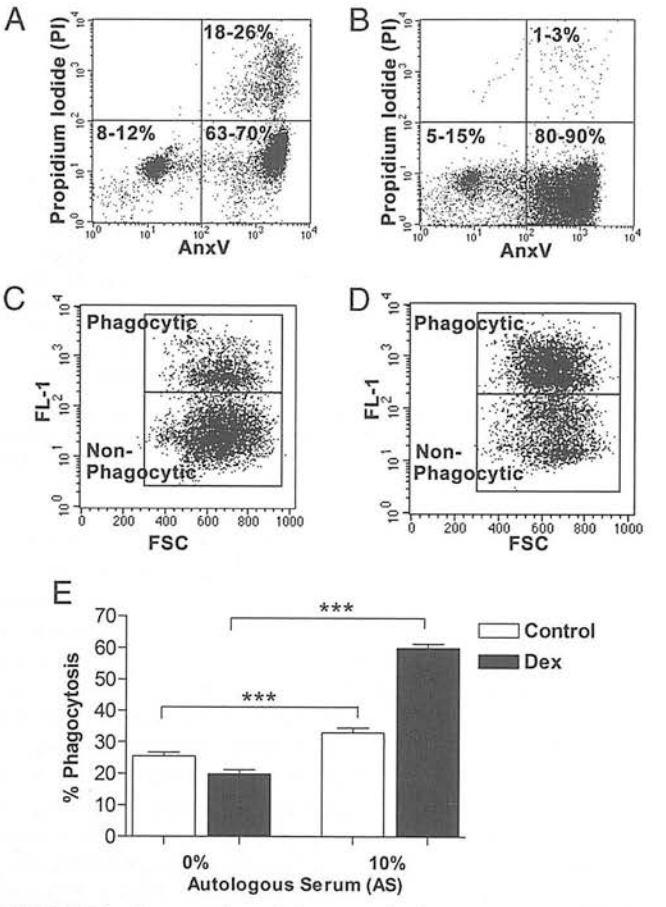

FIGURE 1. Augmentation of phagocytosis of apoptotic neutrophils by glucocorticoid-treated macrophages is serum dependent. Staining with annexin V (AnxV)-FITC (FL-1) and propidium iodide (FL-2) was used to determine the proportion of apoptotic ($AnxV^+$/propidium $iodide^-$) and secondarily necrotic ($AnxV^+$/propidium $iodide^+$) cells present in neutrophil populations used for phagocytosis assays. Representative two-parameter histograms of flow cytometric data are shown for *A*, neutrophils cultured for 20 h in serum-free conditions (n = 35), and *B*, neutrophils cultured for 4 h in serum-free conditions in the presence of 20 μM roscovitine (n = 3). Mean data for the proportion of viable, apoptotic, and secondarily necrotic cells present for each treatment are shown in the appropriate quadrant together with 95% confidence intervals. Phagocytosis of fluorescently labeled neutrophils was determined by flow cytometry using forward scatter and fluorescence to define $FL\text{-}1^{high}$ phagocytic and $FL\text{-}1^{low}$ nonphagocytic MDMϕ populations. Representative dot plots for Dex-MDMϕ incubated with CMFDA-labeled neutrophils in the absence (*C*) or presence (*D*) of 10% autologous serum are shown. FSC, forward light scatter. *E*, Percentage of phagocytosis (±SEM) recorded for untreated MDMϕ (□) and Dex-MDMϕ (■) following incubation with neutrophils for 30 min is shown. Phagocytosis of apoptotic neutrophils by Dex-MDMϕ was significantly augmented by 10% autologous serum (AS). The presence of serum also had a small, but statistically significant stimulatory effect upon phagocytosis by untreated MDMϕ. Data are mean percentage phagocytosis ± SEM, n = 75. ***, $p < 0.001$.

Serum-dependent enhancement of phagocytosis of apoptotic neutrophils does not require complement activation

Down-regulation of complement regulatory molecules CD55 (decay-accelerating factor), CD46 (membrane cofactor protein), and CD35 (CR1) on the surface of human apoptotic neutrophils (31) may allow complement proteins present in serum to bind, and hence promote their removal by phagocytes (11). We found that addition of commercially available C1q-depleted human serum

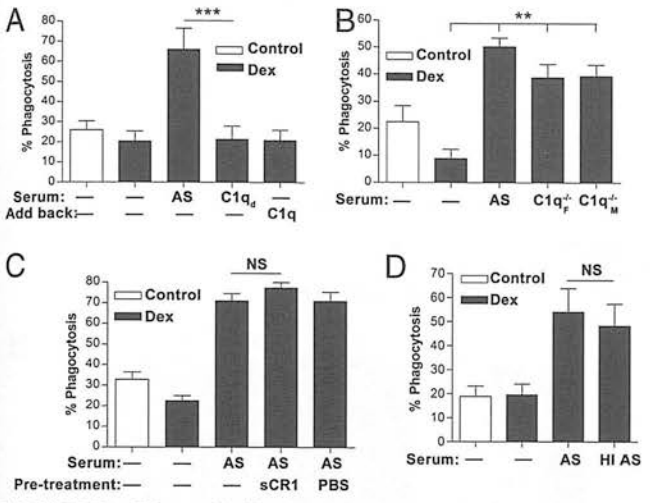

FIGURE 2. Glucocorticoid-enhanced phagocytosis of apoptotic cells does not require complement activation. Phagocytosis of apoptotic neutrophils by untreated MDMϕ (□) and Dex-MDMϕ (■) was assessed in a 30-min assay by flow cytometry. *A*, Phagocytosis of apoptotic neutrophils by Dex-MDMϕ in the presence of serum was not augmented by C1q-depleted serum (C1q$_d$), whereas addition of 70 μg/ml C1q failed to restore augmentation of phagocytosis ($n = 3$; ***, $p < 0.001$ compared with Dex-MDMϕ in the presence of serum). *B*, Serum derived from either male (M) or female (F) C1q-deficient mice restored serum-dependent augmentation of phagocytosis of apoptotic neutrophils by Dex-MDMϕ ($n = 3$; **, $p < 0.01$ compared with Dex-MDMϕ in the absence of serum). *C*, Autologous serum (AS) pretreated with 250 μg/ml soluble human rCR1 to inhibit C3 activation or with PBS as a control for 10 min did not affect phagocytosis of apoptotic neutrophils by Dex-MDMϕ in the presence of serum ($n = 4$; NS), and *D*, heat inactivation of autologous serum (HI AS; 56°C for 30 min) failed to affect phagocytosis of apoptotic neutrophils by Dex-MDMϕ ($n = 4$; NS). Data are mean percentage phagocytosis ± SEM.

failed to confer augmentation of phagocytosis (Fig. 2*A*), suggesting that C1q was the serum opsonin binding to apoptotic neutrophils. However, addition of 70 μg/ml human C1q alone (Fig. 2*A*) or to C1q-depleted serum (data not shown) did not restore phagocytosis by Dex-MDMϕ to levels observed in the presence of autologous serum. In a series of experiments examining the effects of sera from different species, we noted that augmentation of phagocytosis by Dex-MDMϕ was also observed when apoptotic neutrophils were incubated in serum obtained from mice, allowing us to use specific knockouts to define serum components (data not shown). We found that serum derived from either male or female C1q-deficient mice was able to significantly augment phagocytosis of apoptotic neutrophils by Dex-MDMϕ, demonstrating that C1q was not the serum opsonin required (Fig. 2*B*). We next inhibited complement activation and subsequent deposition of C3b on the surface of apoptotic neutrophils by pretreating autologous serum with 250 μg/ml C3 inhibitor for 10 min before the assay. The effects of C3 inhibitor-treated serum were indistinguishable from control PBS-treated serum in enhancement of Dex-MDMϕ phagocytosis (Fig. 2*C*). Further confirmation of a lack of requirement for complement activation and opsonization of targets was made through use of heat-inactivated serum (Fig. 2*D*).

Identification of a serum component required for augmentation of apoptotic neutrophil phagocytosis by Dex-MDMϕ

A number of serum factors have been reported to modulate apoptotic cell phagocytosis by macrophages, ranging from small molecules to very large protein complexes. A series of experiments using size fractionation of serum indicated the serum component to be greater than 100 kDa (data not shown). We initially sought to use an "add back" approach to evaluate the role of well-characterized serum proteins in the observed opsonization phenomenon. This strategy eliminated a role for IgG, pentraxin-3, fibronectin, platelet-derived factors, and immune complexes (Table I). Annexin I and lipoxin A4 are anti-inflammatory mediators that are regulated by glucocorticoids and can act to stimulate phagocytosis of apoptotic cells through the formyl-peptide receptor-like 1 (32). However, pretreatment of Dex-MDMϕ with 10 μM WRW4 (a formyl-peptide receptor-like 1 antagonist) for 1 h before assessment of phagocytosis of apoptotic neutrophils in the presence of 10% autologous serum showed no inhibitory effects (50.5 ± 9.1% and 42.5 ± 6.5% for Dex-MDMϕ with or without pretreatment; mean percentage phagocytosis ± SD, $n = 3$). Similarly, comparison of phagocytosis of apoptotic cells in the presence of either control or annexin I-deficient mouse serum demonstrated that this pathway is not used by Dex-MDMϕ for recognition of apoptotic neutrophils (80.4 ± 6.3% and 79.3 ± 3.23% for Dex-MDMϕ in the presence of wild-type and annexin I knockout serum, respectively; mean percentage phagocytosis ± SD, $n = 3$).

Table I. *Effect of sera and serum proteins on Dex-MDMϕ phagocytosis*[a]

Add Back (during phagocytosis)	Phagocytosis by Dex-MDMϕ (as percentage of phagocytosis by Dex-MDMϕ in the absence of serum)
Autologous serum	301.4
Ultracentrifuged autologous serum	293.8
Murine serum	491.1
Bovine serum	241.8
IgG	109.8
Pentraxin-3	113.4
Fibronectin	97.9
Platelet releasate	84.7

[a] Data are shown as percentage of phagocytosis relative to that recorded for Dex-MDMϕ in the absence of serum (equivalent to 100%) for at least three independent experiments.

Identification of a serum fraction with phagocytic activity

Preliminary experiments showed that the serum factor could be bound to Q Sepharose in a 50 mM HEPES buffer at pH 7.0 or above and eluted with 0.2 M NaCl (data not shown). Because fewer proteins would bind at pH 7.0, we ran subsequent separations at this pH to facilitate identification of the factor. Further fractionation of the 0.2 M NaCl eluate using Sephacryl S-300 column yielded two partially overlapping peaks of protein with descending size (Fig. 3, *A* and *B*), as might be expected for a crude protein fraction with the first peak, representing high molecular mass proteins (>300 kDa), able to confer augmentation of phagocytosis (Fig. 3*C*).

Identification of the serum component using mass spectrometry

Mass spectrometry analysis of the major proteins present in the high molecular mass fraction revealed that the principal proteins present were IgM, α_2-macroglobulin, and C4-binding protein (C4BP), most likely in complex with protein S (33). The presence of protein S in the high molecular mass fractions isolated from gel filtration chromatography was confirmed by immunoblotting analysis (Fig. 3*D*). Previous work had eliminated a role for IgM in the augmentation of phagocytosis of apoptotic cells by Dex-MDMϕ (data not shown). Phagocytosis of apoptotic neutrophils by Dex-MDMϕ in the presence of 20 μg/ml α_2-macroglobulin was not augmented, suggesting that α_2-macroglobulin was not involved either (Fig. 4*A*), whereas addition of purified protein S during the

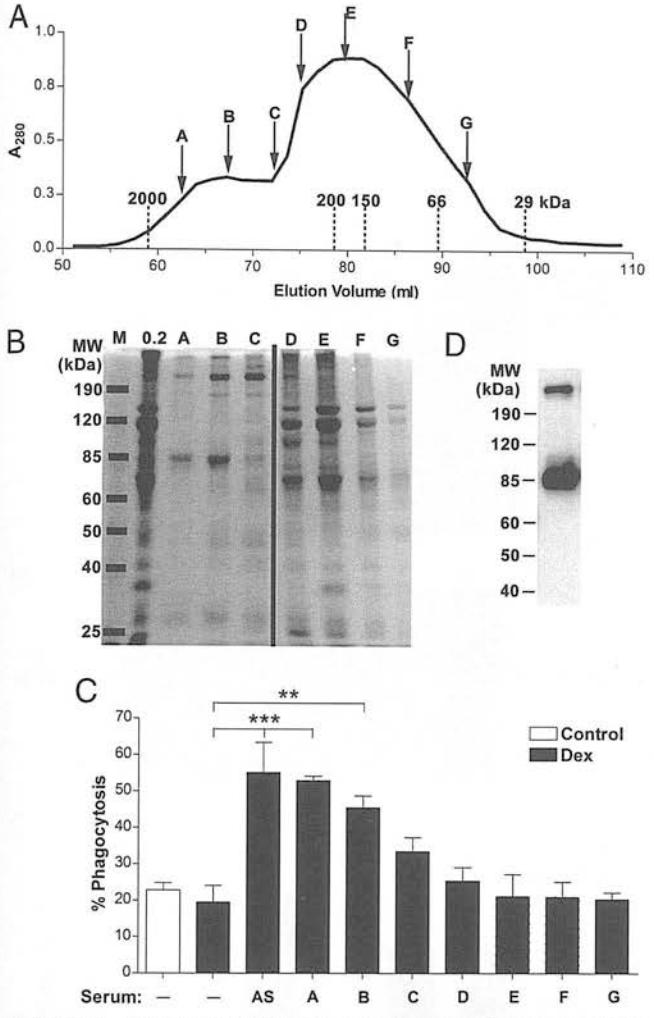

FIGURE 3. Identification of a high molecular mass factor required for augmentation of phagocytosis using anion-exchange chromatography and gel filtration. Serum proteins were fractionated using a combination of anion-exchange chromatography (Q Sepharose) together with gel filtration (Sephacryl S-300) of a 0.2 M NaCl eluate from the Q-Sepharose column. *A*, Protein elution profile of a typical gel filtration separation (of five that were performed) determined by measurement of absorbance at 280 nm (A_{280}) reveals two partially overlapping peaks of protein. *B*, Gel filtration samples (labeled A–G) were separated by SDS-PAGE on a 9% gel under reducing conditions and stained with 0.5% Coomassie blue (M, molecular mass marker; 0.2, 0.2 M NaCl eluate). *C*, The effect of different protein fractions on phagocytosis of apoptotic neutrophils by Dex-MDMϕ (■) was determined using a 30-min phagocytosis assay. Samples were standardized for protein content, and phagocytosis of apoptotic neutrophils by untreated MDMϕ (□) is shown for comparison. Significant augmentation of Dex-MDMϕ phagocytosis of apoptotic neutrophils was observed for two fractions. Data are mean ± SEM; $n = 3$. ***, $p < 0.001$, and **, $p < 0.01$ compared with Dex-MDMϕ in the absence of serum. *D*, The presence of protein S in the high molecular mass gel filtration fraction (fraction A) was confirmed by immunoblotting, as described in *Materials and Methods*.

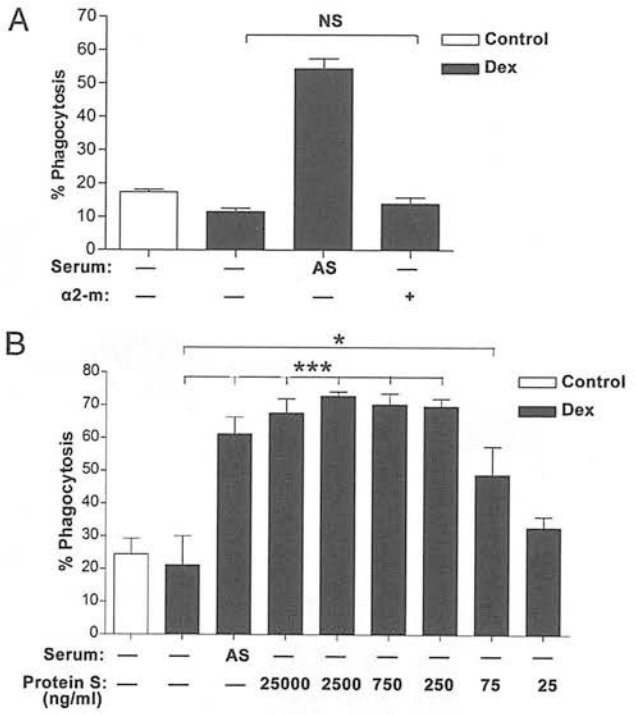

FIGURE 4. Protein S, but not α_2-macroglobulin, stimulates phagocytosis of apoptotic neutrophils by Dex-MDMϕ. The effect of addition of either 20 μg/ml α_2-macroglobulin (*A*) or different concentrations of protein S (*B*) on phagocytosis of apoptotic neutrophils by Dex-MDMϕ (■) was assessed in a 30-min assay. Phagocytosis of apoptotic neutrophils by untreated MDMϕ (□) is shown for comparison. Addition of α_2-macroglobulin failed to restore augmented phagocytosis by Dex-MDMϕ, whereas protein S significantly augmented macrophage phagocytosis at concentrations of 250 ng/ml or higher. Data are mean ± SEM; $n = 3$. ***, $p < 0.001$, and *, $p < 0.05$ and NS, compared with Dex-MDMϕ in the absence of serum.

assay restored phagocytosis of apoptotic neutrophils by Dex-MDMϕ to levels similar to those observed in the presence of 10% serum (Fig. 4*B*). We therefore tested whether immunodepletion of protein S from the 0.2 M NaCl eluate affected Dex-MDMϕ phagocytosis of apoptotic neutrophils. As shown in Fig. 5*A*, immunodepletion did not result in the nonspecific removal of proteins from the 0.2 M NaCl eluate as assessed by total protein staining. Confirmation of the depletion of protein S from the 0.2M NaCl eluate containing the prophagocytic activity was made by immunoblotting (Fig. 5*B*). In contrast to a mock Ab depletion of the 0.2 M NaCl eluate, protein S-depleted 0.2 M NaCl eluate failed to confer augmentation of phagocytosis by Dex-MDMϕ (Fig. 5*C*). Together with data presented in Fig. 3*C*, these data suggested that protein S, possibly complexed with C4BP, was required to confer full phagocytic capacity of Dex-MDMϕ. Interestingly, addition of 250 ng/ml purified human protein S (equivalent to the concentration of protein S present in 1% serum) to protein S-depleted 0.2 M NaCl eluate from ion-exchange chromatography fully restored Dex-MDMϕ phagocytosis (Fig. 5*C*). These data raised the possibility that protein S acts as an opsonin, binding to the surface of apoptotic neutrophils to specifically promote clearance by Dex-MDMϕ.

Protein S binds to apoptotic neutrophils in a calcium-dependent manner to mediate their removal by Dex-MDMϕ

To confirm that protein S was able to opsonize apoptotic neutrophils, we preincubated serum-free apoptotic neutrophils with either the high molecular mass fraction from gel filtration chromatography or 2.5 μg/ml human protein S before washing in IMDM with or without the addition of 5 mM EDTA. Binding of protein S to neutrophils in a Ca^{2+}-dependent manner could be detected by flow cytometry using anti-protein S Ab together with CD16 staining to define apoptotic and nonapoptotic cells (29). The possibility that anti-protein S Abs were binding nonspecifically through FcR-mediated interactions was excluded by use of a rabbit Ig control (Fig. 6*A*) and by mAb blockade of FcγRIIa (data not shown). In the presence of Ca^{2+}, protein S binds to apoptotic (CD16 low-expressing) and also nonapoptotic (CD16 high-expressing) cells, but the

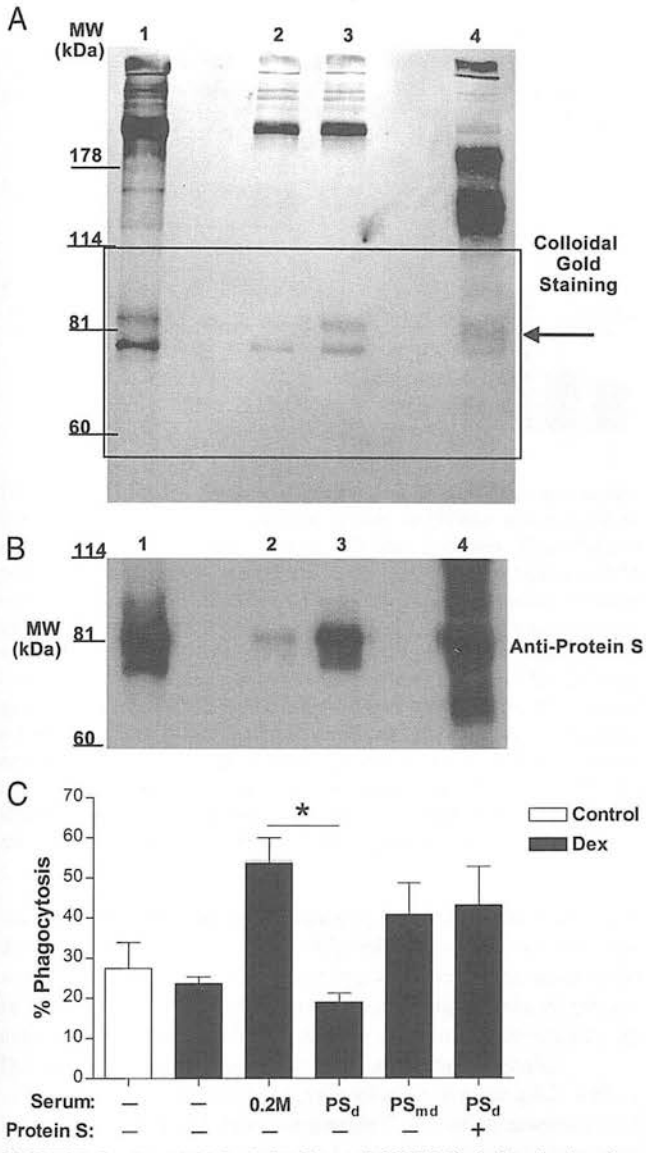

FIGURE 5. Protein S depletion from a 0.2 M NaCl elution fraction from Q-Sepharose abolished augmentation of phagocytosis by Dex-MDMϕ. Immunodepletion of protein S was achieved by three rounds of depletion using a polyclonal rabbit anti-protein S Ab and agarose-coupled goat anti-rabbit IgG, as detailed in *Materials and Methods*. A mock depletion was performed using agarose-coupled goat anti-rabbit IgG alone. Samples were separated by SDS-PAGE on a 9% gel under nonreducing conditions and then transferred to nitrocellulose. *A*, The presence of protein S (~85 kDa) in the 0.2 M NaCl eluate from anion-exchange chromatography and in depleted fractions was confirmed by colloidal gold labeling of transferred protein (*lane 1*, 0.2 M NaCl eluate; *lane 2*, protein S-depleted 0.2 M NaCl eluate; *lane 3*, mock depletion of 0.2 M eluate; *lane 4*, anti-protein S-immunodepleted material, to confirm the presence of protein S). *B*, Specific depletion of protein S in the samples shown in *A* was confirmed by immunoblotting, as described in *Materials and Methods*. In view of the presence of Ab in the immunodepleted sample (*lane 2*), only the outlined section of the membrane in *A* is shown. *C*, The effects of protein S depletion upon phagocytosis of apoptotic neutrophils by Dex-MDMϕ (■) were assessed in a 30-min assay. Phagocytosis of apoptotic neutrophils by untreated MDMϕ (□) is shown for comparison. In contrast to a mock depletion of protein S (PS_{md}), protein S depletion from a 0.2 M NaCl fraction (PS_d) abolished augmentation of phagocytosis by Dex-MDMϕ, an effect that was restored by addition of 250 ng/ml human protein S (equivalent to level in 1% autologous serum). Data are mean ± SEM; $n = 3$. *, $p < 0.05$ compared with Dex-MDMϕ in the presence of 0.2 M NaCl eluate.

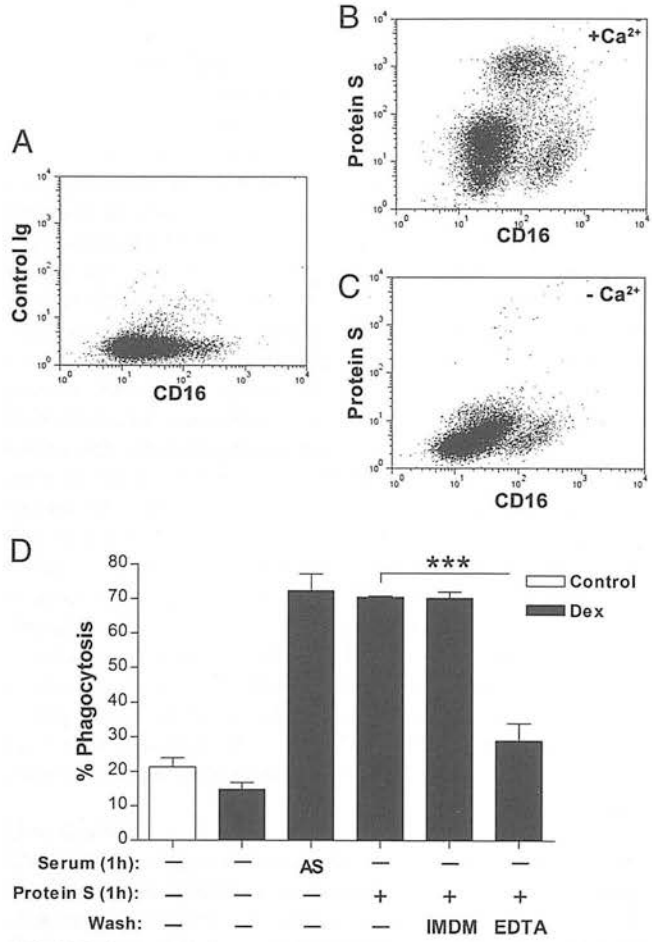

FIGURE 6. Protein S binds to neutrophils in a calcium-dependent manner and confers augmentation of phagocytosis by Dex-MDMϕ. Neutrophils cultured for 20 h in serum-free conditions were preincubated with 1% autologous serum for 30 min in the presence of 1.5 mM $CaCl_2$. During subsequent steps, incubations were performed in TBS in either the presence or absence of 1.5 mM $CaCl_2$ throughout. Neutrophils were washed before labeling with either *A*, rabbit Igs (as control), or *B* and *C*, rabbit anti-human protein S for 30 min. Neutrophils were then washed twice, labeled with PE-conjugated CD16 mAb for 20 min, and washed before flow cytometric analysis. Levels of CD16 expression can be used to define apoptotic (low), secondarily necrotic (intermediate), and nonapoptotic (high) neutrophils. Representative histograms for binding in either the presence (*A* and *B*) or absence (*C*) of 1.5 mM $CaCl_2$ are shown. *D*, Calcium-dependent effects of protein S upon phagocytosis of neutrophils cultured for 20 h in serum-free conditions. Preincubation of neutrophils with either 10% autologous serum or 250 ng/ml purified protein S for 1 h, followed by washing in either the presence (IMDM) or absence (5 mM EDTA) of divalent cations before assessment of phagocytosis of apoptotic neutrophils in a 30-min assay by untreated MDMϕ (□) and Dex-MDMϕ (■). Augmentation of phagocytosis of apoptotic neutrophils by Dex-MDMϕ following protein S preincubation was lost when neutrophils were washed in EDTA-containing IMDM before assessment of phagocytosis. Data are mean ± SEM; $n = 3$. ***, $p < 0.001$ compared with Dex-MDMϕ in the presence of protein S.

level of binding to apoptotic cells was 2.3-fold higher relative to that observed for nonapoptotic cells (Fig. 6*B*). In contrast, protein S binds poorly to cells in the absence of divalent cations (Fig. 6*C*). Interestingly, high levels of protein S binding to secondarily necrotic cells was observed (CD16 intermediate cells; Fig 6*A*), as demonstrated for many other opsonins, including C-reactive protein and C1q (23, 34). Apoptotic neutrophils bind protein S when washed in divalent cation-containing medium, but not when

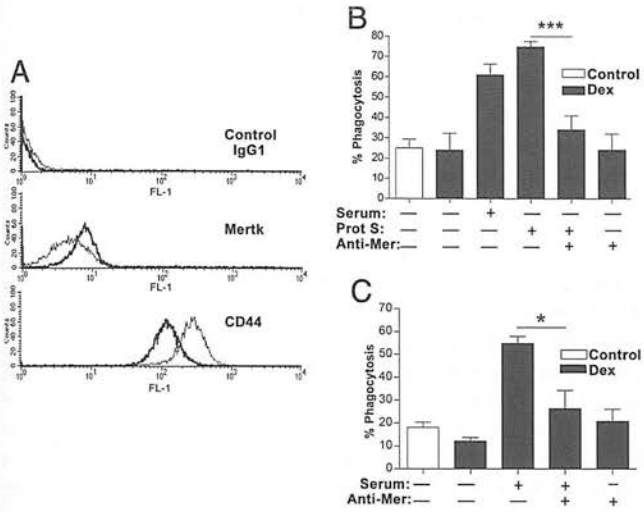

FIGURE 7. Protein S-enhanced phagocytosis by Dex-MDMϕ is dependent on Mertk. *A*, Surface expression of Mer on MDMϕ was assessed by indirect immunofluorescence together with flow cytometry. Representative overlay histograms show expression of Mertk and CD44 for untreated MDMϕ (dotted lines) and Dex-MDMϕ (solid lines) compared with binding of control IgG1 mAb. Expression of Mertk (as determined by mean fluorescence intensity) was increased 1.6-fold in five separate comparisons that were made. In contrast, CD44 expression was down-regulated on the surface of Dex-MDMϕ, as previously reported (7). Preincubation of Dex-MDMϕ (■) with 10 μg/ml anti-Mer Ab for 10 min inhibited subsequent phagocytosis of apoptotic neutrophils in the presence of either 2.5 μg/ml protein S (*B*) or 10% autologous serum (*C*). Pretreatment with anti-Mer alone had no effect on phagocytosis by Dex-MDMϕ in the absence of protein S or serum. Data are mean ± SEM; $n = 3$. ***, $p < 0.001$, and *, $p < 0.05$.

washed in EDTA-containing medium, consistent with a calcium-dependent opsonization event (Fig. 6*D*). However, we also observed low levels of protein S binding to nonapoptotic neutrophils in the presence of divalent cations, suggesting that the prophagocytic effect of protein S on uptake of apoptotic neutrophils by Dex-MDMϕ may require additional cell surface signals.

We also tested the effects of preincubation of neutrophils with or without protein S and then anti-protein S Ab before assessment of phagocytosis. In two experiments that were performed, the results for glucocorticoid-treated macrophages were as follows: no serum (18%), 1% serum (56%), and 1% serum plus anti-protein S (68%). One possibility is that binding of anti-protein S Ab to neutrophils (as shown in Fig. 6, *A–C*) may lead to their opsonization with IgG leading to phagocytosis by FcγR-mediated pathways. In the absence of commercially available Fab′ preparations of anti-protein S Ab to test this possibility directly, we used function-blocking mAb, IV.3, to block the interaction of IgG bound to the neutrophil surface with macrophage FcγRII (CD32). Treatment with IV.3 did not influence phagocytosis (65% phagocytosis for Dex MDMϕ with 1% serum plus anti-protein S; 62% phagocytosis for DexMDMϕ with 1% serum plus anti-protein S in the presence of IV.3; $n = 2$). These data may indicate either that the polyclonal Ab to protein S used in this study does not neutralize the prophagocytic activity or that multiple FcγRs (FcγRIII and/or FcγRI) expressed by MDMϕ mediate the uptake of anti-protein S-opsonized neutrophils.

Protein S-enhanced phagocytosis by Dex-MDMϕ is dependent on Mertk

Surface expression of Mertk, a potential receptor for protein S (35), was increased (1.6-fold) on Dex-MDMϕ compared with untreated MDMϕ (Fig. 7*A*), consistent with previous reports using oligonucleotide arrays (8). The observed up-regulation of Mertk expression was not due to a nonspecific increase in receptor expression because CD44 was decreased on the surface of Dex-MDMϕ relative to untreated MDMϕ (Fig. 7*A*). To assess the contribution of Mertk to protein S-dependent phagocytosis, Dex-MDMϕ were pretreated with an anti-human Mer Ab for 10 min before phagocytosis. Although anti-Mer had no effect on phagocytosis in the absence of protein S, anti-Mer significantly inhibited phagocytosis by Dex-MDMϕ in the presence of 2.5 μg/ml protein S (Fig. 7*B*). Similar experiments were undertaken to determine whether Abs to protein S would exert similar inhibitory effects on phagocytosis. However, pretreatment of neutrophils with anti-protein S resulted in an augmentation of macrophage phagocytosis, possibly through an opsonization event (see Fig. 6) leading to FcγR-mediated phagocytosis. In contrast, blockade of Mer also significantly inhibited phagocytosis in the presence of 10% autologous serum, implying that the Mertk pathway is critical for glucocorticoid augmentation of phagocytosis of apoptotic neutrophils (Fig. 7*C*). We also examined the effects of short-term treatment of MDMϕ with Dex upon the protein S dependency of phagocytosis of apoptotic neutrophils. MDMϕ that had been cultured in the absence of Dex for 96 h were then treated for 24 h with Dex. Compared with untreated MDMϕ (18 ± 5% phagocytosis in the absence of protein S), 96- to 120-h Dex-treated MDMϕ had slightly higher basal levels of phagocytosis of apoptotic cells in the absence of protein S (25 ± 6%), but exhibited increased phagocytosis in the presence of protein S (60 ± 8%).

Discussion

In this study, we have examined the mechanisms that underlie the requirement for serum in augmentation of human macrophage phagocytosis of apoptotic neutrophils following treatment with glucocorticoids. We demonstrated that protein S opsonizes early apoptotic neutrophils (induced by treatment with roscovitine) to promote their internalization by Dex-MDMϕ, and that the presence of cells that had undergone secondary necrosis was not necessary. This is an important observation because a number of serum opsonins have been reported to bind to late apoptotic or secondary necrotic neutrophils, including C1q, and the pentraxins, C-reactive protein and pentraxin-3 (18, 23, 34). Restoration of phagocytic capacity of Dex-MDMϕ by a high molecular mass serum fraction raised the possibility of a requirement for a C4BP-protein S complex, which has been reported to inhibit phagocytosis of apoptotic lymphocyte cell lines (36). Our data showing the presence of protein S in the high molecular mass fraction would imply that the C4BP-protein S complex can augment phagocytosis under some circumstances. Because protein S binding can be demonstrated following incubation of neutrophils cultured in the absence of serum with either the high molecular mass fraction from gel filtration or purified protein S, one possibility is that under certain conditions, protein S can dissociate from C4BP and subsequently oligomerize at the apoptotic neutrophil surface (35). Our data clearly demonstrate that protein S alone is able to confer the augmentation of phagocytosis of apoptotic neutrophils that we observe.

The importance of complement proteins in apoptotic cell opsonization has been highlighted in studies of complement deficiency. In C1q deficiency, impaired clearance of apoptotic cells is thought to contribute to the development of an systemic lupus erythematosus-like autoimmune disease (27). For Dex-MDMϕ, C1q did not restore levels of phagocytosis to those observed in the presence of serum even when C1q-binding proteins such as pentraxin-3 or fibronectin (37, 38) were present. Moreover, C1q-deficient mouse serum was able to confer phagocytic activity, demonstrating that

C1q was not required for efficient phagocytosis of apoptotic neutrophils by Dex-MDMϕ. We were also unable to demonstrate a role for opsonization of apoptotic neutrophils with C3bi for removal through CR3 and CR4, as reported by Elkon and colleagues (11). Furthermore, the data presented in this study argue against a role for IgG, pentraxin-3, fibronectin, annexin I, platelet-derived factors, and immune complexes in phagocytosis of apoptotic neutrophils by Dex-MDMϕ.

We believe that this is the first report demonstrating a switch in the molecular mechanism used by human MDMϕ for apoptotic cell clearance. Our observations are clearly different from the induction of phosphatidylserine-dependent recognition of apoptotic murine thymocytes by bone marrow-derived murine macrophages treated with β1,3 glucan reported by Fadok et al. (39). Treatment with β1,3 glucan did not increase phagocytic potential when compared with untreated bone marrow-derived macrophages, but did alter the molecular mechanism used. In contrast, our findings demonstrate that phagocytosis of apoptotic cells by Dex-MDMϕ is profoundly augmented by glucocorticoids, promoting a critical switch from a protein S-independent to a protein S-dependent recognition pathway.

The tissue microenvironment has the potential to influence the mechanisms involved in apoptotic cell removal and thus apoptotic cell clearance capacity. The cytokine and matrix composition will determine the differentiation status of phagocyte populations during progression of an inflammatory response. Interestingly, protein S-dependent recognition of an apoptotic B cell line was previously characterized in MDMϕ generated by differentiation in M-CSF (35), which promotes the development of M2 macrophages that have anti-inflammatory phenotype properties and respond to TLR stimulation by producing IL-10 (40). We find that when MDMϕ were cultured in the presence of autologous serum, apoptotic cell recognition pathways that are used are predominantly protein S dependent-independent (as shown in Fig. 1). In contrast with dex-treated MDMϕ, these MDMϕ have a more proinflammatory phenotype and release IL-12 in response to TLR stimulation, suggesting that protein S-dependent recognition pathways may be restricted to macrophage phenotypes associated with resolution of inflammation.

The production and release of potential opsonins (complement components, pentraxins, annexins, protein S, etc.) are also regulated during inflammation. A number of reports indicate that inflammation and the coagulation cascade are closely regulated, particularly during the acute-phase response. Protein S is produced in the liver and by endothelial cells (41). Production of both protein S and C4BP in the liver appears to be controlled by inflammatory mediators, including IL-6 (42, 43). Levels of protein S are reduced in patients with ischemic stroke (44) and in patients with sepsis (45), possibly via the effects of TNF on endothelial cells (46). In contrast, glucocorticoids have been reported to elevate levels of protein S (47). Based upon data presented in this work, we propose that a major effect of glucocorticoids on macrophage differentiation is the induction of the capacity to recognize a distinct set of molecular cues that are presented on the apoptotic cell surface. Apoptotic cells display a complex surface molecular signature as a consequence of cell death with altered expression of receptors together with binding (or opsonization) of a number of different proteins. One implication of our observation is that the surface molecular signature of an apoptotic cell may be interpreted differently by different phagocyte populations.

We have also examined the effects of treatment of differentiated MDMϕ with Dex for 24 h (6) upon acquisition of the capacity for protein S-dependent phagocytosis of apoptotic cells. Our data suggest that augmentation of phagocytosis observed following shorter-term treatment is also associated with use of a protein S-dependent pathway for recognition of apoptotic cells. Because both untreated MDMϕ and Dex-MDMϕ populations examined in this study express Mertk, the reason that Dex-MDMϕ are enabled to use a protein S-dependent clearance pathway is not clear. One possibility is that the observed up-regulation of Mertk expression on the surface of Dex-MDMϕ may be sufficient to confer phagocytic potential. Alternatively, Mertk may interact with other receptors on the cell membrane following glucocorticoid treatment. Ligand-activated Mertk forms dimers in the membrane, resulting in Mertk autophosphorylation and activation (48), and may heterodimerize with other Tyro3/Axl/Mer family receptors or cooperate with other receptors involved in the phagocytic process, such as scavenger receptor A (49) or $\alpha_v\beta_5$ (50). Induction of cooperative action of receptors may allow regulation of phagocytosis of apoptotic cells in response to different environmental cues encountered during the inflammatory response.

Alternatively, glucocorticoids may influence engagement of downstream signaling pathways critical for Mertk-dependent phagocytosis. We have previously demonstrated that glucocorticoid-treated MDMϕ exhibited reduced phosphorylation and localization of paxillin and pyk2 to podosome-like adhesion structures, together with increased Rac activity (7). Interestingly, Rac guanine nucleotide exchange factor Vav1 is activated downstream of Mertk (51). Mertk has also been reported to induce FAK phosphorylation and recruitment to $\alpha_v\beta_5$ and formation of p130Cas/CrkII/Dock180 complex (50, 52). One possibility is that phosphorylation of Mertk at Tyr^{867} in the absence of assembly of adhesion structures promotes MDMϕ phagocytic activity (52).

Induced expression of Mertk and protein S by glucocorticoids may promote acquisition of a negative-feedback pathway to both switch off proinflammatory cytokine production (53) and enhance phagocytic capacity for apoptotic cells. The efficacy of glucocorticoids in treatment of autoimmune diseases such as systemic lupus erythematosus that are characterized by impairment of apoptotic cell clearance may be due, in part, to engagement of these pro-resolution mechanisms. Manipulation of the Mertk pathway may represent a novel approach to engage aspects of glucocorticoid action that favor resolution of inflammation without promoting deleterious side effects.

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Disclosures

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Functional and clinical consequences of Fc receptor polymorphic and copy number variants

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Summary

Receptors for immunoglobulins (Fc receptors) play a central role during an immune response, as they mediate the specific recognition of antigens of almost infinite diversity by leucocytes, thereby linking the humoral and cellular components of immunity. Indeed, engagement of Fc receptors by immunoglobulins initiates a range of immunoregulatory processes that might also play a role in disease pathogenesis. In the circulation, five main types of immunoglobulins (Ig) exist – namely IgG, IgA, IgE, IgM and IgD and receptors with the ability to recognize and bind to IgG (Fcγ receptor family), IgE (FcεRI and CD23), IgA (CD89; Fcα/μR) and IgM (Fcα/μR) have been identified and characterized. However, it is astonishing that nearly all the known human Fc receptors display extensive genetic variation with clear implications for their function, thus representing a substantial genetic risk factor for the pathogenesis of a range of chronic inflammatory disorders.

Keywords: Fc receptors, immunoglobulins, polymorphisms, copy number variation, chronic inflammatory diseases

The Fcγ receptor family

Immunoglobulin G (IgG) is the most abundant Ig class in serum, constituting over 75% of circulating immunoglobulin. It mediates key effector functions through interaction with Fcγ receptors, which are encoded by eight different genes, each with multiple transcriptional isoforms and located in a locus on the long arm of chromosome 1 (1*q*21–23). Fcγ receptors are related structurally and belong to the Ig protein superfamily with multiple Ig-like domains. Fcγ receptors are divided generally into three main classes: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), each with distinct structural and functional properties (Fig. 1).

FcγRI is a high-affinity receptor for monomeric IgG (Ka: 10^9–10^{10}/M) with three extracellular Ig-like domains expressed constitutively by monocytes and macrophages, as well as by many myeloid progenitor cells. Three genes coding for FcγRI have been characterized: *FCGR1A*, *FCGR1B* and *FCGR1C* [1]. However, it is generally accepted that only FcγRIa is capable of IgG binding, whereas FcγRIb and FcγRIc possibly represent truncated or soluble forms of the receptor, with poorly characterized function. The α ligand-binding chain of FcγRI associates with a disulphide-bonded dimer of FcR γ chain, a signal transducing polypeptide described originally as a component of the high affinity IgE receptor that associates with various Fc receptors. The γ chain carries an activatory signalling motif (immunoreceptor tyrosine-based activation motif: ITAM), which mediates signal transduction upon FcγRI engagement.

In contrast to FcγRI, the other two classes of Fcγ receptor, FcγRII and FcγRIII, display low affinity for monomeric IgG. They are capable of binding to aggregated IgG through multimeric low-affinity, high-avidity interactions, which are particularly important in the recognition and binding of antibody–antigen complexes during an immune response. IgG binding to low-affinity FcγR can trigger a range of effector and immunoregulatory functions, including degranulation, phagocytosis and regulation of antibody production [2]. Examination of the genomic organization of the FcγRII and FcγRIII locus suggests that these receptors arose as a consequence of multiple gene duplication and recombination processes, followed by gain-of-function mutations. FcγRII is encoded by three genes: *FCGR2A*, *FCGR2B* and *FCGR2C*. All the members of the FcγRII class share a characteristic structure unique to FcγRII, that includes functional signalling motifs in their cytoplasmic domains, namely

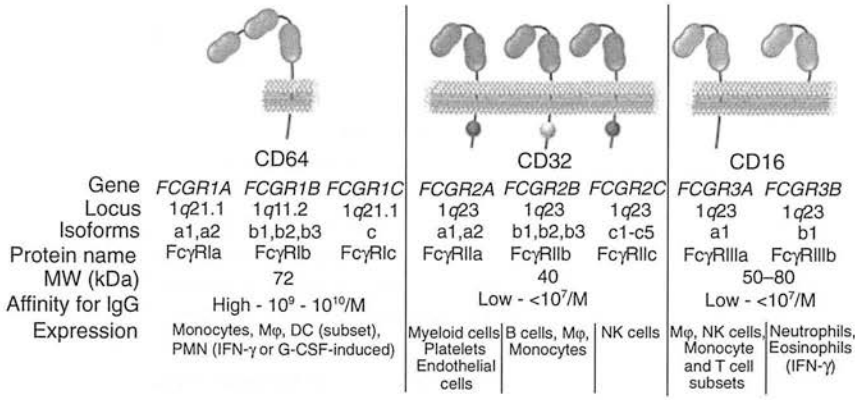

Fig. 1. Overview of the Fcγ receptor family. Mφ, macrophages; DC, dendritic cells; PMN, polymorphonuclear leucocytes; IFN-γ, interferon-γ; G-CSF, granulocyte colony-stimulating factor; NK, natural killer.

ITAM for FcγRIIa and FcγRIIc and immunoreceptor tyrosine-based inhibition motif (ITIM) for FcγRIIb. FcγRII is expressed by diverse cell types: FcγRIIa by myeloid cells, including polymorphonuclear leucocytes, monocytes, macrophages, platelets and certain types of endothelial cells; FcγRIIb by B cells, monocytes and macrophages; while FcγRIIc expression is restricted solely to natural killer (NK) cells.

The other low-affinity Fcγ receptor, FcγRIII, is encoded by genes *FCGR3A* and *FCGR3B*. Although FcγRIIIa and FcγRIIIb share high levels of sequence homology, they exhibit distinct structural differences. FcγRIIIa is a transmembrane protein that associates with the FcRγ chain, whereas FcγRIIIb is processed post-translationally as a glycosylphosphatidylinositol (GPI)-anchored protein, lacking transmembrane and intracellular domains. This difference is thought to be the result of an original gene duplication of the ancestral gene followed by point mutation in the membrane proximal region of the extracellular domain that created a GPI-anchor signal sequence (serine at position 203 rather than the phenylalanine present in the transmembrane FcγRIIIa). The FcγRIIIa isoform is expressed widely by several leucocyte cell types, including macrophages, NK cells and subsets of T cells and monocytes, while FcγRIIIb is expressed constitutively only by neutrophils [2].

The Fcε receptor family

Although normally present in serum at very low levels, IgE may be up-regulated in certain individuals where it associated with atopy, and in immune responses to certain parasites where it is believed to play a protective role. Unlike Fcγ receptors, there are no structural similarities shared by the two classes of IgE receptors: FcεRI, representing the high-affinity receptor for IgE, and FcεRII (CD23), a lower-affinity receptor. FcεRI is a heterotetrameric structure that is comprised of one α subunit, one β subunit and a homodimer of the FcRγ subunit, encoded by *FCER1A*, *MS4A2* and *FCER1G* genes, respectively. The α subunit mediates IgE binding and its structure resembles closely those of the Fcγ receptors, as it consists of two extracellular Ig-like domains with a transmembrane and a cytoplasmic region. The β subunit of FcεRI is a 4-α helix membrane-spanning protein with tyrosine phosphorylation motifs in its C-terminal cytoplasmic region. Recent studies have demonstrated that it exists in two splice variant forms, the β and β_T, the latter having a role in the cellular targeting of FcεRIa [3]. Finally, the γ subunit together with the β subunit facilitates signalling following engagement of FcεRIa. FcεRI is expressed constitutively by mast cells and basophils, whereas monocytes, eosinophils and dendritic cells express FcεRI, but only in its trimeric form (α,γ-γ), lacking the β subunit [3].

FcεRII (CD23) is the low-affinity receptor for IgE, having an affinity of approximately 10^7/M. It is expressed constitutively by B cells and is involved in the regulation of IgE production by B cells – a role related to that of FcγRIIb. In addition, it has been demonstrated that several cell types, including eosinophils, neutrophils, macrophages, monocytes and T cells, up-regulate FcεRII expression upon treatment with interleukin (IL)-4 [4]. FcεRII can also bind and interact with other molecules, such as CD21, CD11b and CD11c, although the functional significance of these interactions is still being defined. FcεRII displays susceptibility to proteolytic cleavage by A disintegrin and metalloproteinase (ADAM) sheddases and soluble FcεRII has been reported to have mitogenic properties [5,6].

Receptors for IgM and IgA

A number of receptors specific for IgA and IgM have been characterized, including the polymeric immunoglobulin receptor (pIgR) and Fcα/μR. These two receptors bind to IgA and IgM with intermediate affinity and are encoded by the *PIGR* and *FCAMR* genes present at the same locus on chromosome 1 (1*q*32). Fcα/μR is expressed by mature B cells and macrophages, as well as in secondary lymphoid organs such as lymph nodes, intestine and appendix, and it is therefore anticipated that it possesses a range of immunoregulatory roles [7]. In contrast, pIgR is expressed predominantly on the basolateral surface of epithelial cells and is involved in the transport of mucosal IgA and IgM across the epithelia [8].

Table 1. Functional effects of genetic variants of Fc receptors.

Receptor	Gene	Variant	Effect	Reference
FcγRIIa	*FCGR2A*	R131H	H131: higher affinity for IgG2	[12]
FcγRIIb	*FCGR2B*	I232T	T232: lower affinity for lipid rafts/decreased inhibitory activity	17,18
		−386G > C	−386C: higher promoter activity	[23]
		−343G > C	−343C: loss of AP-1 binding site/transcriptionally repressed	[24]
		−120T > A	−120A: higher promoter activity	[23]
FcγRIIc	*FCGR2C*	Q57X	Truncated non-functional protein	[21]
		−386G > C	−386C: increased promoter activity	[21]
		−120C > A	−120A: linked with higher transcriptional activity	[21]
		CNV		[21]
FcγRIIIa	*FCGR3A*	V158F	V158: higher affinity for IgG1 and 3, binds IgG4	[14]
		CNV		[35]
FcγRIIIb	*FCGR3B*	NA1/2	NA1: higher affinity for IgG1 and 3	[15]
		SH/A78D	Unknown – linked to the NA2 allele	[16]
		CNV		33,108
FcεRI α chain	*FCER1A*	−66T > C	−66T: higher promoter activity – additional GATA-1 binding site	[26]
		−315C > T	−315T: increased transcriptional activity – Sp1 site	[27]
		−335T > C	−335C: increased expression?	[109]
FcεRI β chain	*MS4A2*	−426T > C	−426C: increased promoter activity	[31]
		−654C > T	−654T: increased promoter activity-YY-1 binding	28,31
		−109C > T	−109T: unknown/higher receptor expression	[28]
		E237G	G237: associated with higher expression	[88]
		I181L	L181: unknown/higher expression?	[88]
		V183L	L183: unknown/higher expression?	[88]
FcεRII	*FCER2*	R62W	W62: resistant to proteolytic cleavage	[5]
pIgR	*PIGR*	A580V	V580: near endoproteolytic cleavage site/reduced efficiency of IgA release?	[8]
FcαRI	*FCAR*	S248G	G248: enhanced IgA-mediated responses; increased cytokine release	[19]

Ig: immunoglobulin.

Although pIgR and Fcα/μR, along with several other molecules, can bind specifically to IgA, the only 'classical' Fc receptor specific for IgA is FcαRI (CD89) [9]. While the ligand-binding (α) chain of FcαRI is related structurally to those of FcγR and FcεRI it is a more distantly related member of the family, and the *FCAR* gene maps to chromosome 19, alongside genes for leucocyte Ig-like receptors and natural killer cell receptors (KIRs). Like many of the FcγR and FcεRI, the FcαRI a chain associates with a homodimer, the FcRγ chain, although it is often expressed in the absence of FcRγ chain pairing.

Fc receptor genetic variation – implications for function

Single nucleotide polymorphisms (SNPs)

The vast majority of the Fc receptor encoding genes display genetic variation either in the form of SNPs or alteration in their copy number. Although many SNPs have been identified for Fc receptors, for most of them their precise impact upon receptor function remains unknown. Functionally relevant genetically determined SNPs can be categorized into three main types, based on the effect they have on receptor function: (i) augmenting the affinity of Fcγ receptors for particular IgG subclasses; (ii) altering the receptor function and consequently downstream effector events; and (iii) affecting transcriptional promoter activity or mechanisms that alter the levels of receptor expression (Table 1).

One of the first functional SNP identified for Fc receptors was the R131H allelic variant for the low-affinity Fcγ receptor FcγRIIa. This point mutation (519G > A) results in an amino acid substitution [arginine (R) to histidine (H)] at position 131, which is located in the membrane proximal Ig-like domain of the extracellular region [10]. Recent crystallographic studies, along with previous mutational analyses, indicated that this region is involved in the receptor interface interacting with the Fc portion of IgG [11] and the R131H variant determines the affinity of FcγRIIa for human IgG2. In particular, while the R131 variant is not able to interact with hIgG2, H131 has been shown previously to bind and enable phagocytosis of hIgG2-coated particles [12]. The H131 variant might be particularly important in conditions characterized by high IgG2 antibody responses, where it may confer enhanced leucocyte activation by IgG2 and increased capacity for clearance of circulating IgG2 complexes.

SNPs affecting the binding affinity for IgG subclasses have also been characterized for FcγRIII. There are two

co-dominantly expressed allelic variants of FcγRIIIa having either a valine (V) or a phenylalanine (F) at position 158. This single amino acid substitution has been demonstrated to increase the affinity of the V158 allotype for IgG1 and IgG3 compared to F158 and induce capacity for IgG4 binding [13,14]. Furthermore, IgG-induced NK cell activity has been found to be increased significantly in 158V/V rather than the 158F/F individuals.

FcγRIIIb is characterized by the presence of the human neutrophil antigen (HNA or NA), a polymorphic variant that comprises four non-synonymous and one synonymous mutation within the membrane distal Ig-like domain of the receptor. The four amino acid differences between the two NA allotypes (NA1 or HNA-1a and NA2 or HNA-1b) have an impact on the N-linked glycosylation of the receptor, and as a consequence affect the affinity for IgG subclasses. In particular, the NA1 allotype displays increased binding and phagocytosis of IgG1- and IgG3-coated particles and it has been shown to exhibit higher affinity for IgG3 compared to the NA2 allotype [15]. Apart from NA-1 and NA-2, the SH polymorphism (also termed as HNA-1c) has been added to the NA family of FcγRIIIb polymorphisms [16]. The SH allele determines the substitution of alanine at position 78 to aspartic acid (A78D) and is linked mainly to the NA2 allele; however, the precise role of this polymorphism in IgG binding is unknown.

In addition to the polymorphisms augmenting receptor affinity for IgG subclasses, SNPs that have a profound impact on the functional effector responses of Fc receptors following immunoglobulin binding have been described. For example, in the case of the inhibitory Fcγ receptor FcγRIIb, it has been demonstrated that a change of a non-polar isoleucine to a polar threonine at position 232 (I232T) within the transmembrane region could affect receptor activity. The T232 variant has been shown to be translocated less efficiently to membrane domains rich in cholesterol and sphingolipids, termed as lipid rafts [17,18]. As a consequence, reduced inhibitory activity was observed for the T232, due possibly to impaired interaction with protein kinases that reside preferentially in lipid raft membrane domains.

A recently identified functional polymorphism in FcεRII is the arginine (R) to tryptophan (W) substitution at position 62 (R62W). In contrast to the R62 variant, the W62 variant is resistant to proteolytic shedding following treatment with a broad range of different proteases, which might influence receptor function and mitogenic role [5]. Finally, a relatively common polymorphism identified in the coding region of FcαRI is associated with impaired capacity to trigger mechanisms such as cytokine release [19]. This polymorphism (844A > G) involves the change of serine 248 to glycine (S248G) within the cytoplasmic domain of the receptor's α chain [20]. IgA-mediated cross-linking of FcαRI on neutrophils from individuals homozygous for the G248 variant triggered significantly more IL-6 release than equivalent cross-linking of receptor on neutrophils from donors homozygous for the S248 variant. In fact, the G248 form, unlike the S248 variant, is capable of inducing cytokine release in the absence of the FcRγ chain. This capacity is presumed to be due, at least in part, to its ability to interact directly with the Src family member Lyn, an important component of the FcαRI signalling cascade.

A number of SNPs have been characterized that play a regulatory role in the expression of particular Fcγ and Fcε receptors. For example, within exon 3 of the *FCGR2C* gene, a single nucleotide substitution at position 202 results in the change of a glutamine residue to a stop codon at position 57 (Q57X), resulting in the production of a non-functional, truncated protein. Furthermore, NK cells from heterozygous 57Q/X donors displayed reduced expression compared to 57Q/Q donors, while homozygous 57X/X donors were negative for NK cell surface receptor expression [21].

Another determinant of the levels of receptor expression are SNPs within the upstream promoter sequences that may affect the transcriptional activity of the promoter. For example, two SNPs, −386G > C and −120T > A, have been identified within the almost identical promoter regions of the genes encoding the FcγRIIb and FcγRIIc receptors [21,22]. Using *in vitro* promoter activity assays, it has been shown that the less frequent −386C and −120A haplotypes exhibit enhanced transcriptional activity compared to the −386G and −120T ones, due mainly to the differential binding of GATA-4 and Yin-Yang 1 (YY-1) transcription factors [22,23]. In addition, genetic linkage was observed between the −386C and −120A alleles. Furthermore, an additional polymorphic site (G to C substitution at the −343 position; −343G > C) within the promoter of the *FCGR2B* gene enables the interaction of the YY-1 transcription factor, leading to transcriptional repression of *FCGR2B* via competition for binding with the c-Jun/AP-1 transcription complex [24,25]. Given the high sequence similarity between the *FCGR2B* and the *FCGR2C* gene promoters, the −343G > C polymorphism would also be predicted to be present within the *FCGR2C* promoter.

Similarly, within the promoter of the gene coding for the α chain of the FcεRI (*FCER1A*), a number of SNPs, including −66T > C, −315C > T and −335C > T, have been shown to regulate receptor expression through differential binding and transactivation of transcription regulatory factors. For example, the −66T variant displayed increased *in vitro* transcriptional activity compared to the −66C form, because the former has an additional GATA-1 binding motif [26]. Similarly, the C to T substitution at position −315 (also referred as −344) was associated with significantly higher promoter activity, an effect that was attributed to the binding of Sp1 transcriptional regulator [27]. Similar SNPs have been identified in the promoter regions of the gene encoding for the β chain of the FcεRI receptor [28–30]. Increased binding of YY-1 and consequently higher promoter activity has been observed for the −426C and −654T haplotypes, compared with the −426T and −654C haplotypes [31].

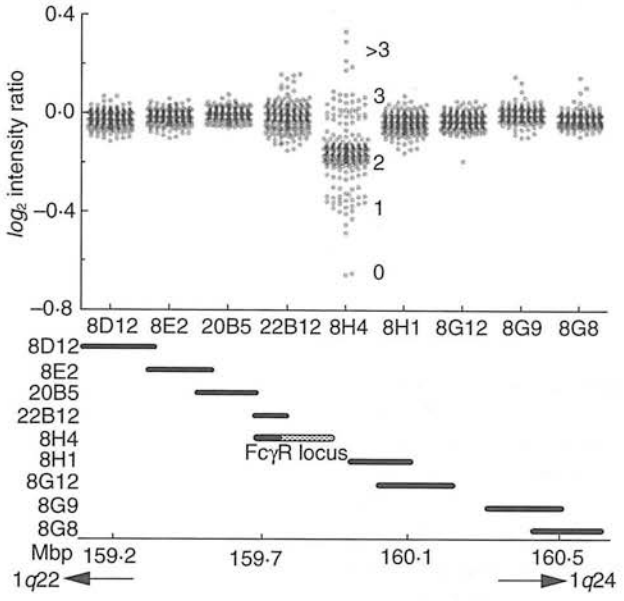

Fig. 2. Copy number variation of the Fcγ receptor locus revealed by genome-wide analysis. Array comparative genome hybridization data from the Whole Genome TilePath (WGTP) project of the Sanger Institute (based on Redon *et al.* [106], available at http://www.sanger.ac.uk/humgen/cnv/data/). Log intensity ratios from 270 HapMap samples for nine probes within the 1*q*23 region of chromosome 1, where the Fcγ receptor locus is mapped. Contrary to other probes, distinct clusters of intensity ratios within the Fcγ receptor probe (8H4) reveal extensive gene copy variation ranging from zero to more than three copies. Similar analyses of the same experimental dataset for the loci where other Fc receptor genes are mapped revealed minimal variation in intensity ratios among the HapMap individuals (data not shown). A notable exception was the FcαR locus (19*q*13) that displays significant variation, due probably to the close proximity of the *FCAR* gene with the *KIR* family genes, which have been described previously to exhibit extensive copy-number variation [107].

Copy number variation

Apart from SNPs, a number of recent studies have demonstrated that genes coding for Fcγ receptors exhibit variation in their copy numbers. Indeed, whole genome scans revealed that over 12% of the human genome is covered by copy number variation, accounting for a great proportion of genetic diversity between individuals (reviewed in [32]). In addition, astonishingly high variation has been noted within the Fcγ receptor locus (Fig. 2), but not within the loci where the genes coding for other Fc receptors are mapped. Copy number variation has been demonstrated for *FCGR3B*, *FCGR2C* and *FCGR3A* genes, but not for *FCGR2A* or *FCGR2B*. For *FCGR3B*, Willcocks and co-workers [33] have demonstrated recently an association between gene copy number and surface expression of FcγRIIIb in neutrophils. In addition, neutrophils isolated from donors with more than two gene copies displayed enhanced IgG-induced effector responses as well as increased cell adherence in IgG-coated surfaces compared with those from donors with less than two [33]. Similarly, NK cells from individuals with two or three copies of *FCGR3A* tend to express higher levels of receptor and exhibit greater antibody-dependent killing capacity than those from individuals with one copy of the gene [21]. Based on these findings, it is anticipated that higher copy numbers of the FcγRIIc gene may be associated with increased levels of surface receptor expression and potentiation of responses following stimulation with IgG. Although a number of studies have made use of well-validated complementary techniques for the assessment of copy number variation, there is controversy on the accuracy and sensitivity of some of these techniques, as they are still at an early stage of technical development.

Fc receptor genetic variants as risk factors for chronic inflammatory diseases

The link between Fc receptor genetic variants and disease pathogenesis has been the subject for intensive investigation for a number of decades and it is accepted widely that they play a crucial role in the pathogenesis of a range of chronic inflammatory diseases, constituting significant genetic risk factors for disease development and prognosis. Based on the functional implications of the Fcγ receptor polymorphic and copy number variants, they can be categorized into either low- or high-responder variants. Low-responder polymorphic variants of Fcγ receptors, for example R131, F158 and NA2 for *FCGR2A*, *FCGR3A* and *FCGR3B*, respectively, as well as a low number of genomic copies, are associated usually with autoimmune pathologies that are characterized by the presence of circulating IgG complexes [34–36]. Reduced efficiency of IgG–Fc interactions with these low-responder variants may compromise clearance of IgG complexes from circulation, leading to their deposition in peripheral tissues; a process that initiates or exacerbates inflammatory processes with detrimental effects. Alternatively, high-responder variants are linked to chronic inflammatory disorders characterized by excessive or inappropriate leucocyte activation [37–39]. These variants may result in more efficient and prolonged Fc–IgG interactions. Reduced threshold for IgG-mediated cellular effector responses could promote leucocyte infiltration into tissues together with the release of histotoxic and cytotoxic compounds that amplify inflammatory cell-mediated tissue damage [40]. A number of chronic inflammatory diseases (summarized in Table 2) have been shown to be associated with Fcγ receptor genetic variants and include (but are not limited to) autoimmune pathologies, such as systemic lupus erythematosus (SLE) [14,17,22,24,25,36,41–60], rheumatoid arthritis [35,61–66], myasthenia gravis [67,68], certain neuropathies[69–72], acute allograft rejection [73] and vascular inflammatory and thrombotic disorders, such as coronary artery stenosis, peripheral atherosclerosis and vasculitis [37,38,74–81].

Table 2. Association of Fcγ receptor variants with chronic inflammatory diseases.

Gene	Variant	Disease	Reference
FCGR2A	H131	GBS	[72]
	R131	Acute renal allograft rejection, APS, giant cell arteritis, HIT, ITP, IgA nephropathy, lupus nephritis, MG severity, peripheral atherosclerosis, RA severity, RF, SLE, WG	[34,36,38,39,41–44,46,47,49,52,57,64,66,68, 73,74,77,80–82,110–118]
FCGR2B	−120A	SLE, CIDP	[22,71]
	−343C	SLE	[24,25]
	−386C	SLE, CIDP	[22,71]
	T232	SLE	[17,53,57–60]
FCGR2C	High CNV	ITP	[21]
FCGR3A	F158	Coronary artery stenosis, giant cell arteritis, lupus nephritis, SLE, WG	[14,37–39,46,50–53,82,83,119]
	V158	ACPA-positive RA, allergic rhinitis, bronchial asthma, HIT, ITP, IgA nephropathy severity, rheumatoid factor production, RA	[35,61–63,65,75,78,110,120,121]
FCGR3B	NA1	ANCA vasculitis, ITP, MG severity	[67,76,79]
	NA2	GBS severity, lupus nephritis, SLE	[55–57,69,70]
	High CNV	ANCA-positive vasculitis	[33]
	Low CNV	SLE, lupus nephritis	[33,86,108]

ACPA: anti-citrullinated protein/peptide antibodies; ANCA: anti-neutrophil cytoplasmic antibodies; APS: anti-phospholipid syndrome; CIDP: chronic inflammatory demyelinating polyneuropathy; CNV: copy number variation; GBS: Guillain–Barré syndrome; HIT: heparin-induced thrombocytopenia; Ig: immunoglobulin; ITP: idiopathic thrombocytopenia purpura; MG: myasthenia gravis; NA: neutrophil antigen; RA: rheumatoid arthritis; RF: rheumatic fever; SLE: systemic lupus erythematosus; WG: Wegener's granulomatosis.

Although there is substantial evidence for the role of Fcγ receptor variants with susceptibility to all these diseases, SLE represents a prototype, multi-organ, antibody-mediated autoimmune disorder, characterized classically by elevated circulating IgG complexes. SLE has been shown to be associated strongly with almost all known Fcγ receptor polymorphic and copy number variants. Several groups have reported an increased frequency of the low-responder allele of FcγRIIIa, F158 among SLE patients [14,46,50–53,82,83]. Furthermore, this allele displays preferential segregation with affected individuals from multiplex SLE families, indicating clearly that it represents a significant risk factor for SLE susceptibility [84]. For FcγRIIa, the R131 allele has been shown to be associated with SLE in several ethnic groups, an observation that was confirmed further by recent meta-analyses [36,49]. Similarly, increased frequency among SLE patients of the low-responder allele of FcγRIIIb, NA2 has been reported widely [55–57]. It should be noted that there have been several reports that describe the lack of association of these polymorphisms with SLE, due possibly to the high genetic variation of these polymorphisms in populations of different origin [50,51,56,85]. In addition, although autoantibodies of all three major subclasses (IgG1, IgG2 and IgG3) can be detected in SLE patients, heterogeneity of the antibody subclass responses and differential clinical exacerbations among these patients could also constitute an additional determinant that accounts for the lack of association between particular Fcγ receptor polymorphisms with disease susceptibility.

A number of polymorphisms within the *FCGR2B* coding and promoter regions that are linked to the development of SLE have been identified. Many of these variants exhibit decreased activity or expression of FcγRIIb that would probably affect B cell function and antibody production, as well as the activity of other cell types such as monocytes and macrophages. A notable example is the transmembrane polymorphism T232, which displays lower affinity for lipid rafts, and as a consequence it exhibits decreased inhibitory activity and has been shown to be associated with SLE, at least in Asian populations [17,53,57–60]. Similarly, the −343C allele that displayed repressed *FCGR2B* promoter activity was enriched among SLE patients, compared to disease-free controls [24,25]. Other SNPs within the promoter of *FCGR2B* are −386G > C and −120T > A, which exhibit genetic linkage. Interestingly, Su and colleagues reported that the frequency of the −386C and −120A alleles, which exhibit enhanced transcriptional activity, was increased in Caucasian patients with SLE compared to ethnically matched controls [22]. The functional relevance of this finding remains to be determined, but one possibility is that these polymorphisms (−386G > C, −120T > A) might be linked genetically to other as-yet unidentified polymorphisms associated with SLE, or their over-representation in the SLE cohort to be due to their low haplotype frequency in the tested population. Finally, variation in the copy number of *FCGR3B* has been identified as an additional determinant for the development of SLE. In two independent studies, low copy numbers of the *FCGR3B* gene was associated strongly

Table 3. Role of IgE, IgA and IgM receptor SNPs in disease pathogenesis.

Receptor	SNP	Disease	Reference
FcεRI α chain	−66T > C	Atopy, high IgE levels in asthma	[26]
	−315C > T	Aspirin-intolerant chronic urticaria, high IgE levels	[98]
	−335T > C	High IgE levels	[109]
FcεRI β chain	E237G	Atopic asthma, high IgE levels, atopy, airway hyperresponsiveness, allergic rhinitis, asthma	[88,90–97,122]
	I181L	Atopy, asthma	[88,89]
	−109C > T	High IgE levels	[28–30,95]
FcαRI	S248G	SLE	[19]
pIgR	A580V	IgA nephropathy	[8]

Ig: immunoglobulin; SLE: systemic lupus erythematosus; SNP: single nucleotide polymorphism.

with SLE, as the percentage of individuals with less than two genomic copies of *FCGR3B* was significantly higher in the SLE compared to the control study cohort [33,86]. Reduced surface expression of FcγRIIIb in these individuals may compromise the clearance of IgG complexes, increasing the risk for the development of SLE.

Apart from Fcγ receptors, polymorphism within FcαRI has been associated with SLE, in that the proinflammatory G248 allele has been found to be enriched in SLE populations [19] (Table 3). In addition, several Fcε receptor polymorphisms have been linked with the development of allergy-related chronic inflammatory diseases, and new associations continue to be reported [87](Table 3). In particular, polymorphisms within the gene coding for the β subunit of the FcεRI (*MS4A2*) have been shown to be associated strongly with atopy, asthma, airway hyperresponsiveness, allergic rhinitis, serum IgE levels, atopic asthma and atopic dermatitis [28–30,88–97]. The extent of the impact of these polymorphisms on receptor function and properties is under investigation. Many atopy-associated SNPs within the promoter of *FCER1A* have been reported to determine transcriptional activity and subsequently receptor expression. In particular, the −66T, −315T and −335C alleles, which differ in their frequency between atopic and non-atopic subjects, at least in some populations, were associated strongly with increased serum IgE [26,27,98]. As the levels of IgE in circulation are correlated greatly with the surface expression of FcεRI, these polymorphisms might affect receptor expression directly or indirectly and consequently the effectiveness of IgE-mediated cellular responses.

In summary, Fc receptors have a key role in the regulation of immune cell function and the polymorphic and copy number variants identified so far undoubtedly contribute to the development of a number of chronic inflammatory diseases. Apart from these diseases, Fc receptor polymorphisms have also been shown to be associated strongly with susceptibility to pathogens, constituting a major genetic risk factor for a number of infectious diseases[99–103]. Furthermore, recent advances in the therapeutic use of intravenous immunoglobulins have highlighted the role of Fc receptor polymorphisms in the clinical outcome and therapeutic responsiveness [104,105]. One of the future challenges is to determine the precise role of the different classes of human Fc receptors in the control of innate and acquired immune responses and define how the observed genetic variation contributes to disease pathogenesis.

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Correspondence

Identification of Fibrocytes in Peripheral Blood

To the Editor:

Dr. Moeller and colleagues have concluded in their article on fibrocytes in idiopathic pulmonary fibrosis (IPF) that fibrocytes comprise more than 10% of circulating leukocytes (white blood cells) in many patients (1). However, the overriding question must be: What do they look like? It would have been a simple matter to use fluorescence-activated cell sorting to separate these collagen 1–positive cells and look at them under a microscope. This would have been a key experiment because in our opinion there has never been any convincing demonstration that fibrocytes can be detected at all in peripheral blood.

In 1994 Bucala and coworkers reported that spindle-shaped cells appeared in adherent populations of human leukocytes after at least 14 d in culture (2). These fibrocytes stained with mesenchymal (connective tissue) markers, as well as the bone marrow stem cell marker CD34. Moeller and coworkers (1) used an antibody recognizing a single mesenchymal marker (collagen 1) to label freeze-thawed, fixed, permeabilized peripheral blood leukocytes from patients with stable and exacerbating IPF. However, the broad spread of laser side-scatter of the "positive" cells suggests that they are not a distinct population of fibrocytes, but rather a mixture of different leukocytes (Figure 1E).

Positive staining with the rabbit polyclonal collagen 1 antibody might have been due to antibody cross-reactivity, aggregation of cells, or Fcγ receptor binding. A polyclonal antibody is actually a mixture of antibody molecules which bind to different protein epitopes. Polyclonal antibodies are prone to bind "nonspecifically" to proteins other than the one used to immunize the animal (collagen 1 in this case). Therefore, positive staining should have been validated by using a panel of antibodies that recognize other mesenchymal and leukocyte markers and CD34. Also, we do not know whether the antibody caused cell aggregation, which could also result in increased fluorescence.

A further problem is that the use of a "control" antibody may be insufficient to control for the fact that antibody preparations contain variable amounts of aggregated IgG, which exhibits high avidity binding to Fcγ receptors on monocytes, granulocytes, and some lymphocytes. This problem can be circumvented by using Fab′ or $F(ab')_2$ antibody fragments which lack the Fc region.

Moeller and coworkers' article suggests that there is something different about the leukocytes of patients with IPF. However, our technical concerns about the data presented in this article indicate that the existence of the enigmatic fibrocytes in peripheral blood remains unproven.

Conflict of Interest Statement: S.B. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.F. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.P.H. received up to $1,000 from Actelion for advisory board activities; he has received lecture fees from GlaxoSmithKline ($1,001–$5,000), and Boehringer Ingelheim (up to $1,000); he has received a grant from Argenta for a clinical trial ($10,001–$50,000).

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From the Authors:

We thank Drs. Bournazos, Fahim, and Hart for their interest in our article (1). In their letter they comment that the "overriding question must be: What do they [fibrocytes] look like?" We want to point out that the presence of fibrocytes in the circulating blood and tissue of animals and humans has been confirmed by several independent groups. Fibrocytes comprise a substantial proportion of circulating leukocytes in pulmonary fibrosis both in human interstitial lung disease (2) and animal models of pulmonary fibrosis (3, 4). In addition, the morphological characteristics of fibrocytes have been published previously, both in cells isolated from the peripheral blood (5) and in the lungs of patients with idiopathic pulmonary fibrosis (IPF) (6). We therefore disagree with the statement that redemonstrating the microscopic appearance of fibrocytes would constitute a "key experiment" for our study.

Side-scatter on flow cytometry is proportional to the internal complexity of cells; as such, the spread of side-scatter in the collagen-1 positive cells is merely indicative of the variability of their internal complexity. We routinely exclude cell aggregates and preblock nonspecific binding with serum and human IgG, as should be done in all flow cytometry staining, so these are not a likely explanation for false-positive (nonspecific) staining. While it is true that affinity-purified polyclonal Abs are a mixture of several Abs that by definition bind different specific Ag epitopes, negative staining with the control affinity-purified polyclonal Abs validates the specificity of the stain. External validation, including staining of fibrocytes with other mesenchymal markers, has been published previously (6). In addition, these cells have been found to stain for procollagens in animal models, further validating their expansion in the context of fibrosis (7).

The key observation in our article (1) was not to demonstrate that the fibrocyte pool is expanded in patients with IPF since this has been shown before. Our intent was to demonstrate that the circulating number of fibrocytes was associated with both exacerbations of IPF and a predictor (biomarker) of mortality in these patients.

Conflict of Interest Statement: M.R.J.K. and J.G. have no financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.M.S. was a paid consultant for Stromedix; he has given lectures at Eli Lilly and Novartis about his laboratory's research. He has an industry-sponsored grant from Novartis related to IL-13 and fibrocytes.

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